Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Pesticides Toxicity Study Using Small Mammals as Bioindicators: Comparison between Conventional and Biological Agriculture

Vânia Isabel da Silva Gaio

Mestrado em Biologia Humana e Ambiente

2010/2011

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Animal

Pesticides Toxicity Study Using Small Mammals as Bioindicators: Comparison between Conventional and Biological Agriculture

Vânia Isabel da Silva Gaio

Tese orientada por:

Professora Doutora Deodália Dias (Departamento de Biologia Animal/Faculdade de Ciências/Universidade de Lisboa)

Doutora Maria da Graça Ramalhinho (Museu Nacional de História Natural/Faculdade de Ciências/ Universidade de Lisboa)

Mestrado em Biologia Humana e Ambiente

2010/2011

Foreword

This thesis is organized into two chapters. In the first chapter, the entire content of the experimental work is presented, while in the second chapter there is presented only the potentially publishable results. The entire thesis is written in English to facilitate that possible publication and the bibliography was made according to the criteria of *Mutation Research*, since it is a potential journal to publish this kind of results.

It is important to note that to carry out part of the experimental work of this thesis, namely to determine acetylcholinesterase inhibition and to quantify manganese, it was necessary to establish a collaboration with Profª Doutora Ana Paula Marreilha dos Santos from Faculdade de Farmácia, Universidade de Lisboa.

Lisboa, October 2011

Vânia Gaio

Acknowledgments

The realization of this thesis would not have been possible without the effort, interest and support of many people, to whom I wish to express my sincere gratitude.

First, I would like to thank my supervisor at Museu Nacional de História Natural from Faculdade de Ciências, Doutora Maria da Graça Ramalhinho, for accepting me as her student and for the constant availability to advice and share her knowledge.

Thanks to Profª Doutora Deodália Dias, my supervisor at Departamento de Biologia Animal from Faculdade de Ciências, for all the support, sympathy and advisement.

I am also very grateful to Profª Doutora Ana Paula Marreilha dos Santos, from Faculdade de Farmácia, Universidade de Lisboa, for your availability, sympathy and suggestions which helped to enrich the content of this thesis.

I want to thank Vanda Andrade and Dinamene dos Santos for their patience, technical support and their precious help with Atomic Absorption Spectrometry.

To Joaquim Tapisso, Sofia Gabriel, Margarida Duarte, Patrícia Sardinha, Rita Dario and Susana Ferreira for all the help, support and friendship you gave me. Thank you for all the support in the laboratory and kindness.

I am also very grateful to Ana Sofia Boa-Alma, my lab partner, who has always accompanied me in this work.

Thanks to the farmers, who collaborated in this study, for their support in the field and for always being available to provide all the necessary information.

Final words go to my family for always encouraged me and give me all the emotional support. Special thanks to my boyfriend, who always believe in me. Luís, I could not finish it without you. Thank you for all the strength you gave me through difficult times!

Resumo

A poluição ambiental induzida por substâncias químicas é considerada um grave problema a nível mundial e o uso generalizado de pesticidas é, talvez, o tipo de poluição química mais preocupante da actualidade, afectando todo o planeta, incluindo a saúde humana. A agricultura é uma importante actividade na economia de muitos países, mas é, ao mesmo tempo, a grande responsável pelo aumento do consumo de pesticidas a que temos vindo a assistir, ao longo dos tempos. Em Portugal, de acordo com o Eurostat, aproximadamente 17000 toneladas de pesticidas foram vendidos em 2008, contrastando com menos de 13000 toneladas vendidas em 1997.

Pode considerar-se que os pesticidas são uma ferramenta económica, eficiente e racional no controlo de pragas pois, teoricamente, são desenvolvidos através de processos de regulamentação muito rígidos para que funcionem com segurança e mínimo impacto na saúde humana e no ambiente. No entanto, a maioria das vezes, os estudos publicados não estão em concordância com este facto e surgem, cada vez mais, preocupações relativamente aos riscos para saúde, decorrentes não só da exposição ocupacional aos pesticidas, mas também da exposição a resíduos destes químicos, presentes nos alimentos, que vão atingir toda a população. Consequentemente, nos últimos anos, procuram-se alternativas viáveis ao uso de pesticidas e a agricultura biológica pode ser uma opção a considerar. De facto, este tipo de agricultura não usa qualquer tipo de produto químico, sendo baseada na utilização de inimigos naturais para o combate de pragas. Todavia, esta prática é muito controversa, pois parece não ter ainda o potencial para responder a uma produção em massa, necessária para alimentar toda a humanidade. Assim, é essencial que se faça mais investigação nesta área de forma a entender as verdadeiras potencialidades, constrangimentos e limitações desta prática, que pode ser a chave para a resolução do grande problema do uso de pesticidas.

O Mancozeb é um dos fungicidas mais usados a nível mundial, principalmente devido à sua baixa toxicidade aguda para os mamíferos e escassa permanência no meio ambiente. Apesar disto, o Mancozeb, principalmente através de um dos seus produtos de degradação, a etilenotiureia (ETU), tem efeitos toxicológicos ao nível da tiróide, sistema imunitário, reprodutivo e nervoso. Para além disto, o Mancozeb parece ter actividade mutagénica, teratogénica e ainda carcinogénica, tendo-se

verificado que induz uma variedade de tumores de diferentes origens em ratos, após uma exposição crónica. Há ainda a referir que o manganês (Mn), constituinte deste pesticida, também é um agente mutagénico comprovado, causando danos no material genético. Apesar de tudo isto, o Mancozeb está incluído na lista (Anexo I da directiva 91/414/EEC, 1991) de substâncias activas consideradas aceitáveis, tendo em conta o seu impacto no ambiente, saúde animal e humana, sendo o seu uso legalmente autorizado na formulação de pesticidas dentro da Comunidade Europeia.

A avaliação ambiental da toxicidade dos pesticidas pode ser feita através da utilização de pequenos mamíferos roedores, que vivem nos campos, uma vez que estes são bons bioindicadores, acumulando um vasto espectro de poluentes presentes nos ecossistemas. Particularmente em relação aos pesticidas, existem já algumas correlações significativas entre exposição e a ocorrência de danos genéticos nestes animais. Por outro lado, estes animais têm a vantagem de ter grandes semelhanças genéticas e fisiológicas com os humanos, permitindo a extrapolação dos efeitos dos pesticidas.

Assim, o principal objectivo deste trabalho foi avaliar a toxicidade dos pesticidas, *in situ*, comparando o estado de saúde de duas espécies de roedores (*Mus spretus* e *Apodemus sylvaticus*), provenientes de uma zona de agricultura convencional em contraste com os de uma zona de agricultura biológica. Para isto, foi necessária a colaboração dos agricultores em causa, que se mostraram sempre disponíveis, e forneceram toda a informação necessária. Assim, o agricultor responsável pelo campo de agricultura biológica garantiu que não eram usados quaisquer tipos de substâncias químicas, tratando-se de uma zona que sofreu conversão há cerca de 5 anos. Da agricultura convencional foi fornecida uma lista de pesticidas usados, onde os insecticidas organofosforados, carbamatos, juntamente com alguns EDBCs eram os principais grupos químicos de pesticidas usados. Para além disto, o agricultor da zona convencional informou que mais de 50% das aplicações eram baseadas num fungicida EDBC, o Mancozeb, sendo então este o pesticida maioritariamente aplicado.

Depois de capturados e sacrificados, os animais foram avaliados do ponto de vista morfofisiológico, através das medidas biométricas, peso relativo dos órgãos internos e parâmetros hematológicos. Foram ainda sujeitos a três testes de forma a avaliar os danos ao nível do material genético: ensaio do cometa nos linfócitos, teste dos micronúcleos na medula óssea e teste das anomalias nos espermatozóides.

Para além disto, foi feita a determinação da actividade da acetilcolinesterase no cérebro, que é um biomarcador da exposição a insecticidas organofosforados e carbamatos, e a quantificação do manganês (Mn) no fígado, que pode servir para avaliar a exposição ao Mancozeb, uma vez que este metal entra na sua constituição. Foi, ainda, iniciada uma análise molecular no intuito de determinar polimorfismos do gene *CYP1A1,* que está envolvido no processo de desintoxicação de agentes xenobióticos, como os pesticidas.

Como resultado, o presente estudo demonstrou claramente que a exposição a uma mistura de pesticidas, onde o Mancozeb representa mais de 50% dessa mistura, tem a capacidade de causar danos genéticos em pequenos mamíferos, avaliados pelos ensaios do cometa, micronúcleos e anomalias nos espermatozóides. Para além disto, de forma a provar que, de facto, o Mancozeb era o agente genotóxico em causa, foi feita a quantificação do manganês no fígado dos animais, através da técnica de Espectrofotometria de Absorção Atómica com Câmara de Grafite. Assim, concluiu-se que os animais de ambas as espécies da zona da agricultura convencional, expostos a pesticidas, com maiores níveis de danos genéticos, tinham também maiores níveis de Mn no fígado, comparativamente aos animais da zona de agricultura biológica. Ou seja, o Mancozeb, sendo a fonte destes níveis de Mn mais elevados, será o agente genotóxico em causa.

Por outro lado, foi feita a avaliação da actividade do enzima acetilcolinesterase, no cérebro, para despistar possíveis efeitos de insecticidas organofosforados e carbamatos, que também podiam ser a causa das diferenças nos danos genéticos encontrados entre as duas zonas analisadas. No entanto, verificou-se que não havia diferenças significativas nos valores da actividade deste enzima, entre os animais das duas zonas, que pudessem justificar os valores superiores de danos genéticos encontrados na zona de agricultura convencional.

Foi ainda feita uma análise morfofisiológica para verificar se os animais expostos aos pesticidas tinham esses parâmetros alterados, mas apenas os baços aumentados foram reveladores da infecção provocada pela exposição aos pesticidas. Por outro lado, foi feita uma análise genética preliminar, no intuito de encontrar polimorfismos no gene *CYP1A1*, mas não foi possível conclui-la e, futuramente, será necessário continuar esta abordagem molecular do estudo.

Tendo em conta as diferentes sensibilidades das duas espécies bioindicadoras analisadas, este estudo mostra como é importante usar pelo menos

duas espécies simpátricas para detectar a presença de agentes genotóxicos e utilizar vários testes de avaliação dos danos genéticos, porque a variabilidade em espécies selvagens é bastante mais elevada relativamente a animais de laboratório. Por outro lado, verificou-se que, de facto, a quantificação do Mn, tem potencial para ser um biomarcador da exposição e este tipo de pesticidas, que têm metais na sua constituição. Este estudo fornece ainda valores de referência para os biomarcadores utilizados em estudos futuros e demonstra como *M. spretus* e *Apodemus sylvaticus* são duas espécies indicadas para serem usadas como bioindicadores de poluição provocada pelos pesticidas.

Concluindo, o presente estudo demonstra que a prática agrícola convencional representa um maior risco genotóxico comparativamente à agricultura biológica e contribuiu para alertar acerca dos efeitos prejudiciais que advêm da exposição aos pesticidas a longo prazo, particularmente, da exposição ao Mancozeb, que é um pesticida de uso legalmente autorizado na Comunidade Europeia. Apesar de vários estudos indicarem uma elevada toxicidade do Mancozeb, este continuará a ser um dos pesticidas mais usados no mundo, devido á sua eficácia, baixo custo e baixa persistência no ambiente relativamente a outros pesticidas. Assim, grande parte da população vai continuar a ser exposta e a problemática dos seus efeitos a longo prazo continuará a existir, enquanto não forem feitos investimentos na descoberta de alternativas ao uso de pesticidas.

Palavras-chave: Pesticidas, Mancozeb, *Mus spretus*, *Apodemus sylvaticus*, Biomarcadores de Genotoxicidade, Manganês, Acetilcolinesterase

Abstract

Pesticides are widely used for pest control in conventional agriculture, despite their negative impacts. Biological agriculture can represent a valuable option, avoiding the use of pesticides, but it does not appear to have the potential to respond to the mass production of food to feed humanity.

Mancozeb is a widely used fungicide mainly because of its low acute toxicity in mammals and scarce persistence in the environment. However, it has been considered a multipotent carcinogen, mutagenic and possibly teratogenic in a long-term exposure context.

Small mammals can be used as bioindicators of environmental toxicity of pesticides, accumulating a wide spectrum of pollutants, and significant correlations between pesticides and genetic damage in free-living rodents have already been detected.

The main aim of this study was the *in situ* assessment of pesticide toxicity, using two small mammals' species (*Mus spretus* and *Apodemus sylvaticus*) from a conventional agriculture (CA) versus a biological agriculture (BA) zone.

It was found that exposure to a mixture of pesticide, where Mancozeb represents more than 50% of that mixture, has the ability to cause DNA damage in small mammals, assessed by comet, micronucleus and sperm abnormality assays. Moreover, both species from CA zone, exposed to pesticides, with greater genetic damage, have also greater levels of Manganese. Thus, considering that Mancozeb is the widely used pesticide in the CA zone, we can conclude that this pesticide will be the source of the highest levels of manganese found in small mammals and it is the genotoxic agent at issue. Moreover, acetylcholinesterase activity revealed that organophosphates and carbamates insecticides also applied are not involved in that genotoxic action.

In conclusion, the present work contributes to alert about hazard effects resulting from pesticide exposure, particularly Mancozeb, a pesticide legally authorized for use in European community.

Key words: Pesticides, Mancozeb, *Mus spretus*, *Apodemus sylvaticus,* Biomarkers of Genotoxicity, Manganese, Acetylcholinesterase

Table of Contents

Figures Index

Tables Index

List of Abbreviations and Symbols

AAS - Atomic absorption spectroscopy;

AChE - Acetylcholinesterase;

ACT- Acetylthiocholine;

ALS - Alkali labile sites;

AO - Acridine orange;

BA - Biological agriculture;

bp - Base pair;

CA - Conventional agriculture;

DBCP - Dibromochloropropane;

DDT - Dichlorodiphenyltrichloroethane;

DMSO - Dimethyl sulfoxide;

DNA - Deoxyribonucleic acid;

DSB - Double-strand breaks;

DTCs - Dithiocarbamates;

DTNB - Dithiobisnitrobenzoate ion;

EBDCs - Ethylenebisdithiocarbamates;

EDTA - Ethylenediaminetetraacetic acid;

EPA - Environmental Protection Agency of United States of America;

ETU - Ethylenethiourea;

Eurostat - Statistical Office of the European Communities;

FAO - Food and Agriculture Organization of the United Nations;

FISH - Fluorescent in situ hybridization;

GFAAS - Graphite furnace atomic absorption spectrometry;

GSH - Glutathione;

GSSG - Glutathione disulfide;

Hct - Hematocrit;

Hgb - Hemoglobin;

IARC - International Agency for Cancer Research;

LC_{50} - Median lethal concentration;

LD_{50} - Median lethal dose;

MCH - Mean corpuscular hemoglobin;

MCHC - Mean corpuscular hemoglobin concentration;

MCV - Mean corpuscular volume;

Mn - Manganese;

MN - Micronucleus;

MNPCE - Micronucleated polychromatic erythrocyte;

NaCl - Sodium chloride;

NaOH - Sodium hydroxide;

NCE - Normochromatic erythrocytes;

OPs - Organophosphates;

PAHs - Polycyclic aromatic hydrocarbons;

PCE - Polychromatic erythrocyte;

PCR - Polymerase Chain Reaction;

RBC - Red blood cells;

RNA - Ribonucleic acid;

ROS - Reactive oxygen species;

RS - Reactive species;

SNP - Single nucleotide polymorphism;

SSB - Single-strand breaks;

TNB - 5-thio-2-nitrobenzoate anion;

Tris - Tris(hydroxymethyl)aminomethane;

UNEP - United Nations Environment Programme;

WBC - White blood cells;

WHO - World Health Organization;

Zn - Zinc.

Chapter I

1. General Introduction

1.1. The Pesticide Problem

The environmental pollution induced by the chemical substances is regarded as a serious problem. Particularly, the widespread use of pesticides is affecting the entire planet, including the human health [1-3].

According to FAO (Food and Agriculture Organization of the United Nations), a pesticide is defined as any substance or mixture of substances intended for preventing, destroying or controlling any pest. This includes vectors of human or animal diseases, unwanted species of plants or animals that cause harm during the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs [4]. Given this broad definition, is not surprising that pesticides include a wide range of different substances that are commonly used for different proposes.

It is recognized that World War II was responsible for the development of various pesticides that we employ at the present. In fact, some pesticides currently in use were developed for application in warfare, like organophosphates used as nerve gases. After World War II, this chemicals began to be used as pesticides in environmental spraying for mosquito eradication and in agriculture even when their potential hazards were unknown [5].

However, during the 1960s and 1970s, it began to emerge some evidences that these chemicals could have harmful consequences. Epidemiologists in the United States of America noted a rise in the incidence of blood cancers and when plotted on a map, these cases were clearly clustered in agricultural areas. This increase in blood cancers incidence paralleled the increase in pesticide use, has led some epidemiologists to assume that there was a causal link [5] and, in 1962, with the first publication of the Rachel Carson's revolutionary book, *Silent Spring* [6], it was started the slow process of raising political and public consciousness of the effects of pesticides in wildlife, humans and ecosystems.

As a consequence of these first evidences of pesticides hazards, by the end of the 1960s, it was created the United States Environmental Protection Agency (EPA)

that was the first governmental agency taking action against pesticide usage. Subsequently, in 1972, United Nations began to recognize the risks posed to humans and to the environment by the increased usage of pesticides and decide to establish the United Nations Environment Programme (UNEP) which together with World Health Organization (WHO) and FAO promoted more sustainable agricultural practices like integrated pest management [7]. Furthermore, all over the years, there have been other efforts and initiatives to improve regulation of international pesticide trade and an example is the creation and update of an International Code of Conduct on the Distribution and Use of Pesticides [8].

Nevertheless, the world has known a continuous growth of pesticide usage, not only in number of different chemicals used but also in quantities sprayed [9]. In the European Union there are more than 800 of synthetic pesticide products with multiple formulations [10] and the trend is an increase because mostly pests develop resistance and, therefore, chemical companies continuously synthesize new ones. According to Eurostat, Statistical Office of the European Communities [11], roughly 17000 tones of pesticides were sold in Portugal, in 2008, while in 1997 was less than 13000 tones (Figure 1). Although sales declined after 2002, that trend has apparently reversed, with substantially increasing in 2007 and 2008.

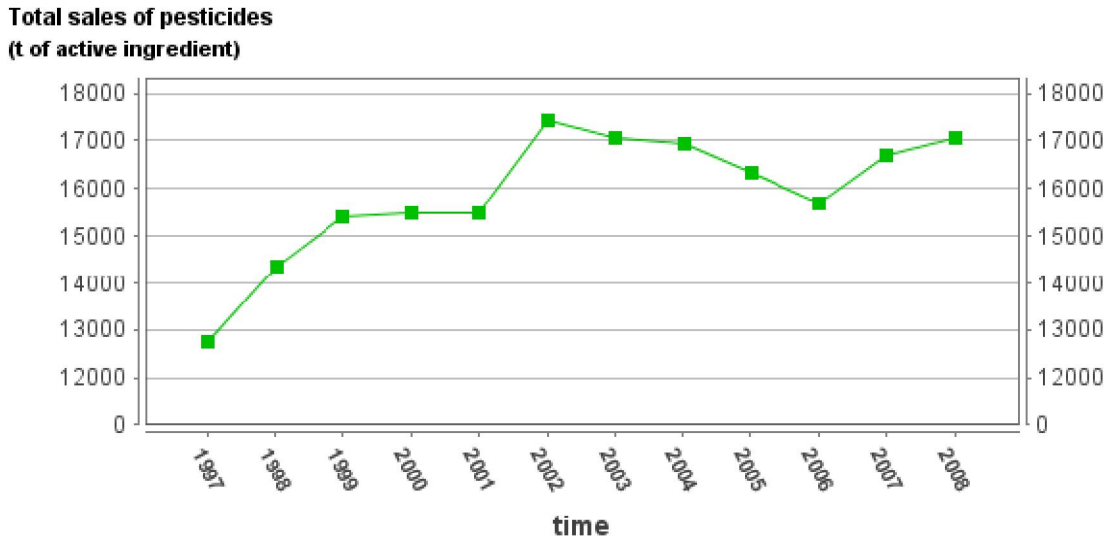

Figure 21. Total volume of pesticides sold in Portugal. The total is the sum of fungicides, herbicides, insecticides and other pesticides [11].

Pesticides refer to chemical substances that are biologically active and interfere with normal biological processes of living organisms deemed to be pests. They include a wide range of compounds and according to their functional class of

organisms designed to control, they can be classified as insecticides, fungicides, herbicides, rodenticides, molluscacides, nematocides and others that belonging to different chemical groups. They are deliberately spread into the environment, both in urban and rural areas, for industrial, agricultural and public health purposes and, after application, residues may persist in the environment, contaminating soils and water, remain in the crops, enter the food chain, and finally they are ingested by humans with foodstuffs and water [9, 12].

About their persistence in the environment, pesticides can be classified as organochlorines, such as DDT, that are considered persistent pesticides because they have long environmental half-lives, being transported over long distances by global circulation [13] and tend to bioaccumulate in humans and other animals, [14, 15]. On the other hand, contemporary pesticides, such as organophosphates, carbamates, triazines, and others have much shorter environmental half-lives [16] and tend not to bioaccumulate. Even so, humans are chronically exposed to many of these no persistent chemicals because they are widely used in agriculture in large quantities which enhance their hazardous effects.

At the present time, agriculture is an important activity and source of economic income in several countries all over the world but, at the same time, it is largely responsible for the increased consumption of pesticides [17]. Indeed, pesticides used in agriculture are very important to reduce yield losses, maintain high product quality and sometimes improve the nutritional value of food and its safety. From this point of view, pesticides can be considered as an economic, labor-saving, and efficient tool of pest management [18]. In addition, pesticides are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment [8, 19, 20]. Even though the published results are not always in agreement with this and serious concerns have been raised about health risks resulting not only from occupational exposure to pesticides but also from pesticides residues on food and in drinking water for the general population [10].

Consequently, in the last years, additional efforts have been produced to reduce the use of pesticides and biological agriculture can represent a valuable option. In Europe, the biologically managed land area has continued to grow (Figure 2) and almost 7.8 million hectares (1.9 percent of the agricultural land) were managed biologically by more than 210000 farms in 2007 [21].

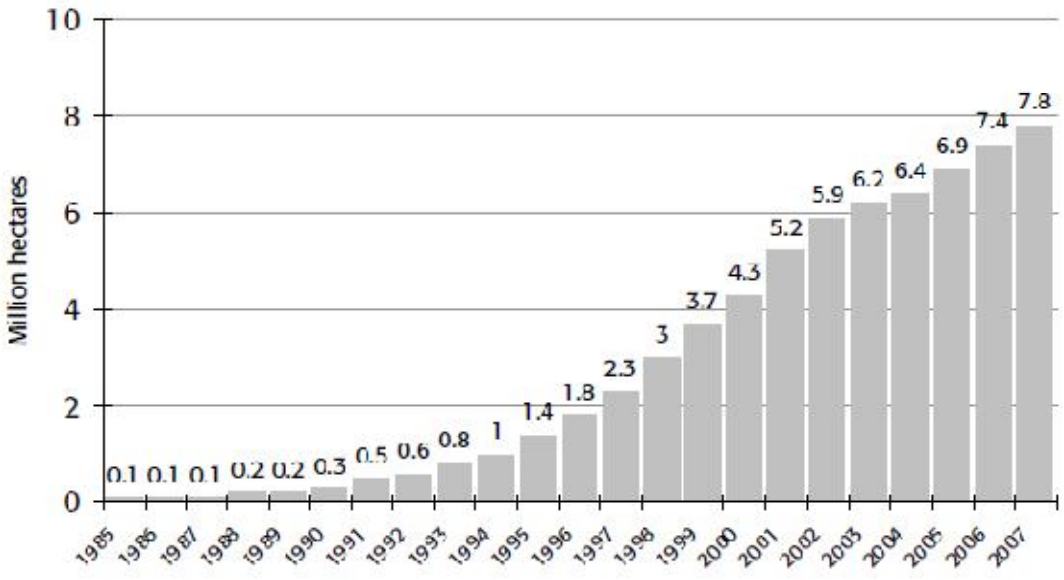

Figure 22. Development of the biologically managed agricultural land area in Europe between 1985 and 2007 [21].

This practice respects the normal functioning of ecosystems, avoiding the use of pesticides, and leads to food ‘‘free’’ of synthetic chemicals. It has grown so much in the last 20 years that it corresponds to the use of millions of hectares already [22]. Nevertheless, organic agriculture does not appear to have the potential to respond to the mass production of food to feed humanity [9] and it deserves wide experimentation to fully understand its potentialities, constraints and limitations [23].

Given the current problem of pesticide usage, an increase of pesticide legal actions, like regulations, directives, decisions and recommendations for the use of pesticides is very important but not sufficient. It is urgent to find and develop alternative and profitable agricultural practices, in order to minimize the consumption of pesticides, which represents a real global problem.

1.2. Pesticides Toxicity and Potential Health Effects

As a consequence of the widespread use of pesticides, all people are inevitably exposed and concern is growing in the international community about the health effects consequences to this ubiquitous contamination [12].

WHO and EPA have already classified pesticides according to their potential health risks. According to these classifications (Table 1 and 2), pesticides are grouped based on their median lethal dose (LD_{50}), which is the pesticide dose that is required to kill half of the tested animals when entering the body by oral or dermal way [24].

Table 2. Acute toxicity of pesticides according to WHO classification (adapted from [24]).

Class	Classification	LD_{50} for the rat (mg/Kg body weight)			
		Oral		Dermal	
		Solids	Liquids	Solids	Liquids
Ia	Extremely hazardous	<5	<20	<10	<40
Ib	Highly hazardous	5-50	20-200	10-100	40-400
II	Moderately hazardous	50-500	200-2000	100-1000	400-4000
III	Slightly hazardous	>501	>2001	>1001	>4001
U	Unlike to present acute hazard	>2000	>3000	-	-

Table 2. Acute toxicity of pesticides according to EPA classification (adapted from [25]).

Class	Signal words	Acute toxicity for the rat		
		Oral LD_{50}(mg/Kg)	Dermal LD_{50}(mg/Kg)	Inhalation LC_{50}(mg/L)
I	Danger	<50	<200	<0.2
II	Warning	50-500	200-2000	0.2-2.0
III	Caution	500-5000	2000-20000	2.0-20
IV	Caution	>5000	>20000	>20

The oral LD_{50} is usually lower than the dermal LD_{50} because pesticides can enter to the bloodstream more easily through the stomach than through the skin [26]. Moreover, according to WHO classification, there are differences for liquid and solid pesticides. In fact, the toxicity of the liquid formulation is usually much higher than that of the respective solid formulation since it is more difficult for a solid to pass through the skin [27]. In addition, the acute inhalation lethal concentration (LC_{50}),

which is the pesticide concentration required to kill half of the exposed tested animals, is also considered in EPA classification. Despite all the known risks of some of these pesticides, many of those catalogued as extremely hazardous and dangers are still being used in some regions of the planet.

While data on the acute toxicity of many of pesticides is easily recognized, knowledge on their delayed effects is much more limited. In particular, pesticide residues in vegetables and fruits continue to generate concern in the general population mainly about their potential long term adverse effects such as cancer. These long-term effects resulting from a regular intake of pesticide are hard to detect and quantify [10, 28].

Efforts have been made to assess carcinogenicity of pesticides and the International Agency for Research on Cancer (IARC), an international organization that is part of WHO, has already a classification based on evidences derived not only from experiments with animals but also from epidemiological studies in humans and other relevant studies where carcinogenicity of pesticides is tested [29]. Accordingly, a pesticide is classified in different groups as presented in Table 3.

Table 3. Classification of Pesticides carcinogenicity according to IARC [29].

Group	Classification
1	**Carcinogenic to humans**
2A	**Probably carcinogenic to humans** - limited evidence of carcinogenicity in humans but sufficient evidence in test animals.
2B	**Possibly carcinogenic to humans** - limited evidence of carcinogenicity in humans and less than sufficient evidence in test animals.
3	**Not classifiable as to its carcinogenicity to humans** - inadequate evidence of carcinogenicity in humans and inadequate or limited evidence in test animals.
4	**Probably not carcinogenic to humans.**

EPA has a similar classification where the respective classes are carcinogenic to humans; likely to be carcinogenic to humans; suggestive evidence of carcinogenic potential; inadequate information to assess carcinogenic potential; and not likely to be carcinogenic to humans [30].

It is important to note that these classifications results mainly from toxicological studies on experimental animals and from epidemiological studies and not result from cause-control studies on humans. In addition, toxicological testing is

based on the administration of a single active ingredient, whereas human exposures are to a complex mixture of compounds. So, these classifications should be interpreted with extra caution because they can be associated with high uncertainty in the estimation of the relevant human exposure pattern [31]. On the other hand, when there is lack of toxicity data for specific pesticides, this does not necessarily imply that they are not toxic. They may have not been evaluated yet or that relevant data are not available. In truth, we need to know much more about the environmental behavior of these chemicals and about their effect upon humans.

It is essential to invest in research of pesticides effects and, in fact, there are hundreds of scientific studies done on all continents to determine if there is a relationship between pesticide use and human health problems [32-35]. These studies have reported associations between exposure to pesticides and various health outcomes including immunologic abnormalities [1, 36], adverse reproductive and developmental effects [37], cancer [35, 38], and neurological diseases, such as Parkinson`s [39] and Alzheimer's diseases, where cholinesterase inhibition may play an important role [40, 41].

DNA damage and oxidative stress have been proposed as mechanisms that could link pesticide exposures to these health outcomes [42] and may be involved in the pathogenesis of various diseases including cancer. In fact, exposure to a wide range of pesticides induces oxidative stress reflected as accumulation of reactive oxygen species (ROS), lipid peroxidation and DNA damage [43]. Mechanisms leading to these alterations in cellular redox homeostasis are only partially understood for certain pesticides but, in general, pesticides have been shown to alter cellular redox balance by their enzymatic conversion to secondary reactive products and/or ROS, by depletion of antioxidant defenses and by the impairment of antioxidant enzyme function [44, 45].

Additionally, for environmental stressors, such as pesticides, ROS may serve as common mediators of programmed cell death (apoptosis) and pathological conditions [46]. In conditions of homeostasis, cell duplication is compensated by the cell death, but in pathological conditions there is a deregulation (excessive or deficient) cell death and homeostasis in not maintained. Apoptosis in one of this mechanisms of cell death that is highly regulated and it is characterized by the progressive activation of precise pathways leading to specific biochemical and morphological alterations [47]. When induced by environmental toxicants, such as

pesticides, apoptosis is widely associated with alterations in redox homeostasis which include both the depletion of antioxidant defenses, like Glutathione (GSH) and the increase accumulation of reactive species (RS) of oxygen or nitrogen (Figure 3). This will originate a direct damage of the mitochondria or indirectly via activation of death receptor, endoplasmic reticulum stress and/or DNA damage [43, 46]. So, apoptosis can be the mechanism involved in pesticide-induced pathologies where cell death has a major role.

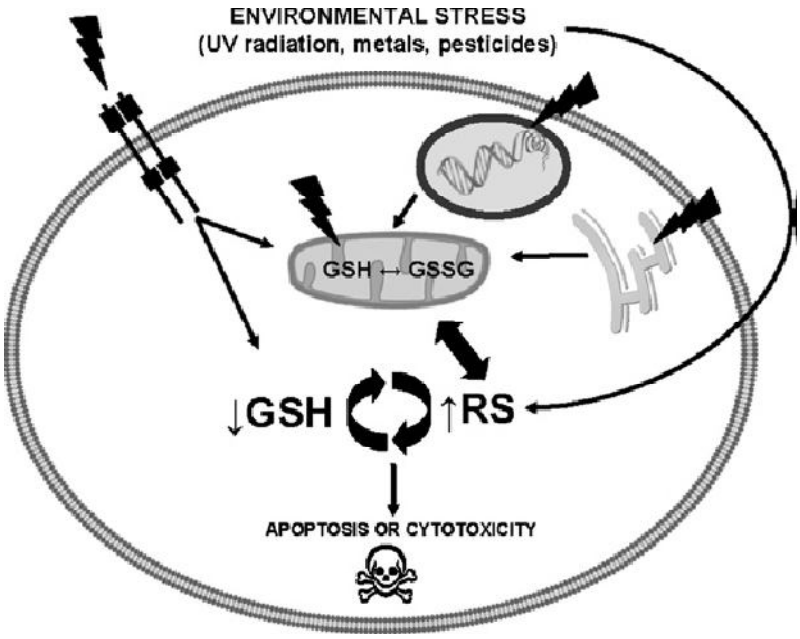

Figure 23. Schematic representation of apoptosis induced by environmental stressors. Apoptosis can be induced by activating intrinsic pathways (mitochondria, DNA damage and/or endoplasmic reticulum stress) as well as by extrinsic pathways (activation/modulation of death receptors). When induced by environmental toxicants it is widely associated with alterations in redox homeostasis which include both the depletion of antioxidant defenses (such as GSH) and the increase accumulation of reactive species of oxygen or nitrogen. GSH-Glutathione; GSSG-Glutathione disulfide; RS-Reactive species [48].

Furthermore, various experimental data have provided evidence that pesticides are potential chemical mutagens inducing gene mutation, chromosomal alteration and DNA damage [10]. These genotoxic effects are also considered serious of the possible side effects of pesticides because they may produce DNA breakage at sites of oncogenes or tumor suppressor genes, thus playing a role in the induction of malignancies in individuals exposed to these agents [49].

1.2.1. The Particular Case of Mancozeb

Among the 25,000 types of pesticides available, more than 80% are organophosphates or carbamates. Analogs of these last ones, dithiocarbamates, where oxygen atoms are replaced by sulfur atoms, are widely used as fungicides to protect fruits, vegetables and field crops against a large spectrum of fungal diseases. Particularly, ethylenebisdithiocarbamates (EBDCs) which include Maneb, Zineb, and Mancozeb are regularly used in agriculture [50].

Mancozeb (Figure 4), a greyish-yellow powder, is a polymeric complex of EBDC-Manganese with zinc salt (molecular weight of 265.3 + 65.4) and it is one of the most widely used commercial fungicides worldwide [51], mainly because of its low acute toxicity in mammals and scarce persistence in the environment [52]. It is not known to occur as a nature product and it is synthesized from carbamate radicals that have reacted with carbon disulfide to give dialkyldithiocarbamates. After that, it reacts to diamines originating dithiocarbamates, and, finally, with the addition of a zinc chloride, is produced Mancozeb [29, 53].

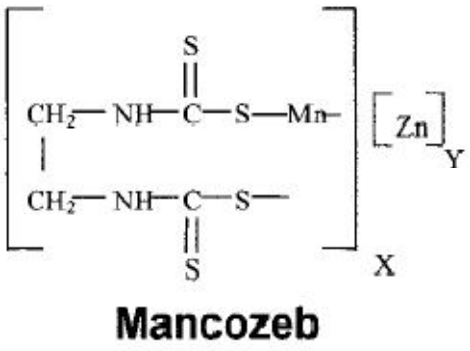

Figure 24. Chemical structure of Mancozeb [51].

Mancozeb is used to control fungal diseases that afflict many important economic crops, including potato, tomato, fruits and flowers. It is a broad-spectrum pesticide that indiscriminately kills a range of organisms, targeted as well as untargeted (and beneficial) species. It is a contact fungicide (non systemic) that disrupts cell metabolism, namely lipid metabolism [54]. This synthetic fungicide has a short environmental resistance with low solubility in water and low soil persistence. It is hydrolyzed within 1 day in sterile water [53] and has a field half-life of 1 to 7 days. On the other hand, it has low volatility at standard temperatures and pressure but can be found associated with air-borne particulates or as spray drift.

Even though Mancozeb has been characterized as less acutely toxic and less persistent in the environment, one of its main degradation products resulting of a spontaneous breakdown, ethylenethiourea (ETU) shows a long persistence (5–10 weeks) in soil and high water solubility. ETU is thought to be the source of most of the toxicity associated with EBDCs and consequently with Mancozeb [50]. Its residues are regularly detected in fruit and vegetables, and it has been shown that a significant percentage of ETU is produced during cooking of contaminated foods [55]. This compound has been reported to have carcinogen, teratogen and goitrogen effects [53, 56, 57].

Although the risk of intoxication by EBDCs mainly concerns industrial and agricultural workers [58], the general population can be chronically exposed to dietary residues present of such pesticides in food. Mancozeb, despite its low acute toxicity, has been shown to produce significant toxicological effects on thyroid [58, 59], immune system [60], reproductive system [61-64] and nervous system [65], Moreover, Mancozeb was considered a multipotent carcinogen, inducing a variety of tumors of different origin in rats after chronic exposure [66], mutagenic [67] and possibly teratogenic [68].

The alkylation of DNA is a mechanism that could be invoked to explain the DNA damage induced by the EBDC pesticides [69]. On the other hand some studies demonstrate that Mancozeb may act as prooxidants inside the cell. This mechanism is based on the presence of coordinated transitional metals, like manganese and zinc present in the chemical structure of this pesticide that will catalyze the formation of ROS through the Fenton reaction, where metals have a strong catalytic power to generate highly reactive radicals [67, 70].

Even though Mancozeb is considered to have a low acute toxicity in mammals (Class U in the WHO classification) [24] and not listed about its carcinogenicity in IARC classification, it is crucial to give a little attention to their long-term effects not only from the harmful action of ETU but also from its metallic constituents.

1.3. Using Biomarkers to Assess Pesticides Toxicity

The term biomarker is used to include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical, or biological [71]. In other words, biomarkers are indicators of events in biological systems that could clarify relationships between environmental hazards and health effects or disease process [72]. Therefore, biomarkers can be used to identify causal associations, making possible to recognize susceptible groups or individuals who are at risk of exposure to certain toxic agents [73].

Biomarkers can be divided into three classes (Figure 5): biomarkers of susceptibility, that indicate an organism`s inherent or acquired limitations affecting its response to a toxic exposure; biomarkers of effect, that provide information on an preclinical event occurring at a target site in consequence of exposure, correlated to manifestation of disease; and biomarkers of exposure, that integrate all routes of exposure to a particular contaminant [74, 75].

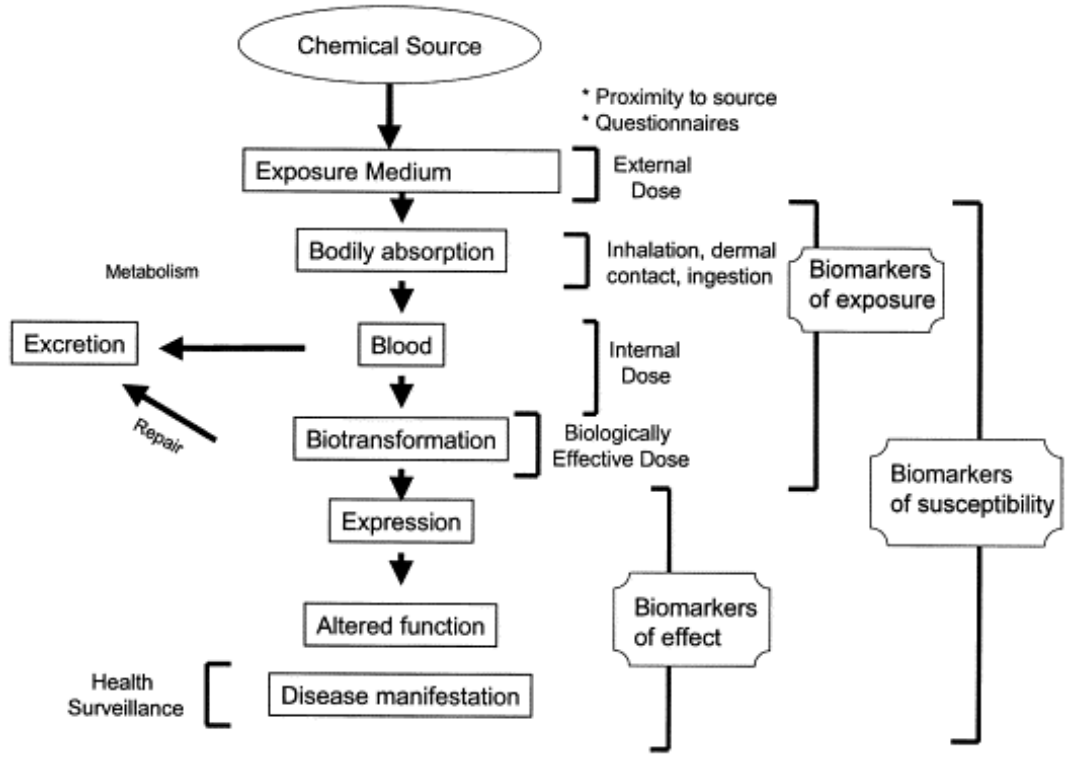

Figure 25. Schematic representation of the different types of Biomarkers of a toxicant from exposure to induction of health effects. [75].

Additionally, biomarkers of exposure currently available for monitoring pesticide exposure can be divided into two main groups: internal dose and biologically effective

dose [74]. Internal dose means the measurement of chemical agents or their metabolites either in tissues, secreta, excreta or exhaled air in order to evaluate exposure and health risk [76]. A biomarker of biologically effective dose measures the amount of a toxicant that has interacted with a target site and altered its physiological function. These biomarkers may be spontaneously repaired or may lead to the development of disease [75, 76].

The use of several biomarkers is recognized as an important approach for the assessment of pollution, as chemical analysis of environmental samples alone does not provide evidence of the impacts in organisms. Biomarkers to assess pesticides toxicity cover a wide range [28] but this study will focus only on some biomarkers of genotoxicity (Comet, Micronucleus and Sperm Abnormality assays), acetylcholinesterase inhibition, manganese quantification and one biomarker of susceptibility (*CYP1A1*).

1.3.1. Biomarkers of Genotoxicity

Biomarkers of genotoxicity provide measures of the alterations of important genetic targets, like chromosomes, chromatids or gene sequences where the link between the measured parameter and the development of tumors is probably closer [77]. Examples are sister chromatid exchanges, micronuclei, comet assay and chromosome aberrations, that are often associated with higher cancer incidence [78].

For genotoxic chemicals, such as pesticides, techniques that measure DNA damage provide a powerful tool in measuring environmental effects and the most commonly used test for genetic damage is classical chromosome aberration analysis. However, an inexpensive and simpler test looks for micronuclei, whole or fragmented extra chromosomes resulting from abnormal mitosis. Some studies use, yet, newer tests to assess DNA damage, such as Sister Chromatid Exchange, Comet assay and Fluorescent in situ hybridization (FISH), assay [79]. Sperm abnormality assay is also regularly applied to get information on the transmission of genetic damages. The use of multiple tests is very useful because it provides more information about the different types of lesions in the genetic material, allowing for greater reliability of results.

1.3.1.1. Comet Assay

The Comet assay or single-cell gel electrophoresis test is a useful technique for studying DNA damage and, during the last two decades, it has become a method of increasing popularity [80-82]. It has been accepted by the United Kingdom Committee on Mutagenicity testing of chemicals in food, consumer products, and environment as a test for assessing DNA damage [83].

Ostling and Johanson (1984) were the first to use this assay to quantify DNA damage induced by radiation in cells. Their technique consisted of lysing cells with detergents and high salt, and subjecting the liberated DNA to electrophoresis under neutral conditions. Cells with an increased frequency of DNA double-strand breaks displayed increased migration of DNA toward the anode. The migrating DNA was, then, quantified by staining with ethidium bromide and by measuring the intensity of fluorescence using a microscope photometer. The amount of the DNA liberated from the head of the “comet” depends on the effect of the genotoxic agent [84].

However, the neutral conditions used by Ostling and Johanson greatly limited the general utility of the assay because it only detects double-strand breaks (DSB) [85]. Consequently, the assay was adapted under alkaline conditions by Singh *et al*. (1988), which led to a sensitive version of the assay because it could assess both double and single-strand DNA breaks (SSB) as well as the alkali labile sites (ALS) expressed as single-strand breaks [86].

The alkaline version of the Comet assay (Figure 6) is the methodology of choice. Generally, a cell suspension is embedded in agarose on a microscope slide and lysed by detergents and a high salt concentration in order to liberate the DNA. Afterwards, the slides are treated at alkaline conditions (pH>13) to produce single stranded DNA and to express ALS as SSB. During electrophoresis at the same alkali condition, DNA fragments induced by genotoxic agents migrate to the anode side. Subsequently, the slides are washed with a neutralizing solution, where DNA strands separated by alkaline treatment in the comet head will readily renature due to their intact structure with supercoiled loops, while the DNA in tail will remain single-stranded [81, 82]. Then, slides are stained with and fluorescent DNA binding dyes and when viewed under a microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments [87]**.**

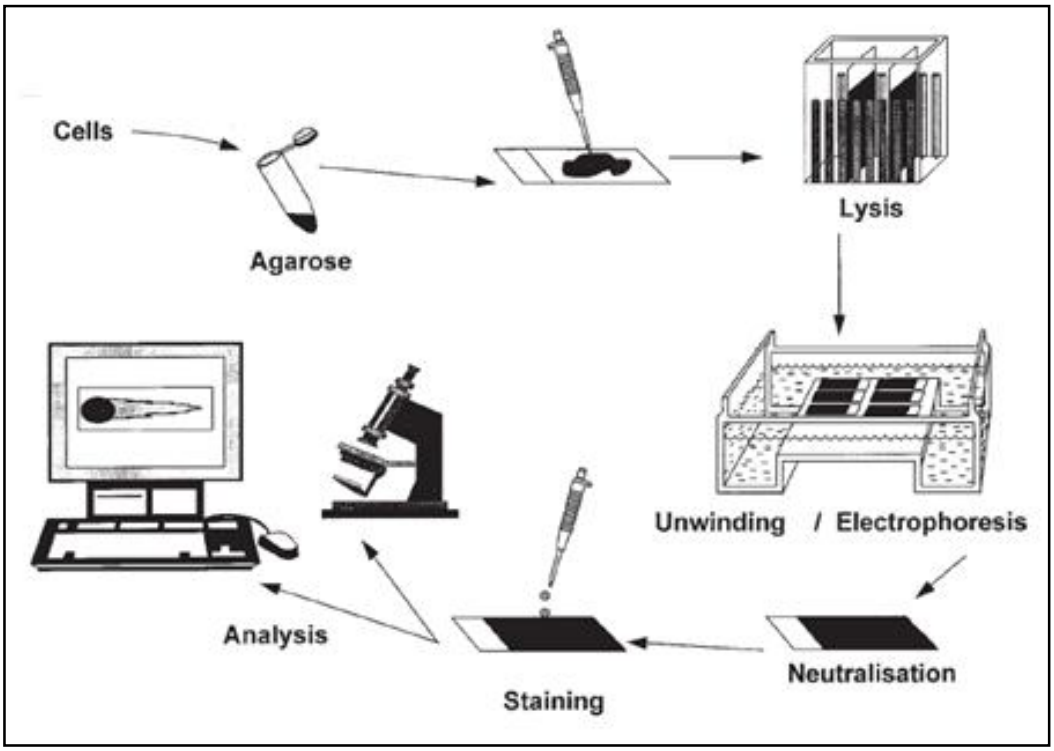

Figure 26. Schematic representation of critical steps in the alkaline version of Comet Assay. Adapted from Speit and Hartman, 1999 [88].

Scoring of DNA damage can be performed by visual (manual) scoring or by using an automated software scoring system with an image capture device attached to the microscope, that will permit the measurement of parameters such as the percentage of DNA in the tail (% tail DNA), tail length and tail moment (fraction of migrated DNA multiplied by some measure of tail length). % tail DNA is considered a better parameter as this gives a clear indication of the appearance of the comets and, in addition, is linearly related to the DNA break frequency over a wide range of levels of damage. It represents a more sensitive measure of DNA damage because it continues to increase with increased DNA damage, while tail length may not [89-93].

The Comet assay possesses a number of advantages as compared to other genotoxicity tests. It is sensitive for detecting low levels of DNA damage, applicable to various tissues and/or special cell types, flexible to use proliferating as well as non proliferating cells, require for only small numbers of cells per sample, easy of application, low cost, and one short time needed to perform the assay [85]. This is a non-invasive technique compared to other DNA damage techniques, such as chromosomal aberrations or micronucleus assay in the bone marrow, which require a larger sample as well as proliferating cell population [81]. In addition, this assay can be used to evaluate various types of DNA damage and is readily modifiable for

adaptation to a variety of experimental requirements [85]. DNA damage assessed by the Comet assay gives an indication of recent exposure and at an early stage where it could also undergo repair without resulting in permanent genetic alteration [94]. Thus, it provides an opportunity for intervention strategies to be implemented timely [83].

A limitation of the Comet assay is that aneugenic effects, which may be a possible mechanism for carcinogenicity and epigenetic mechanisms of DNA damage such as effects on cell-cycle checkpoints, are not detected. On the other hand, neither the extent of DNA migration in the comet assay nor the shape of the comet can reveal the mode of action of the mutagenic potential of a test substance and it has technical variability and interpretation [95]. However, the advantages far outnumber the disadvantages, and hence, it has been widely used in fields ranging from molecular epidemiology to genetic toxicology [83].

1.3.1.2. Micronucleus Assay

The Micronucleus (MN) assay applied to erythrocyte cells has been usually used as a simple and sensitive method for determining the genotoxicity of chemical substances [96, 97]. It is extensively used as a biomarker of chromosomal damage, genome instability, and eventually of cancer risk [98]. This assay is based on the detection of small nuclei (micronuclei) formed during the metaphase-anaphase transition of cell division. It may be a chromosome (aneugenic phenomenon leading to loss of chromosome) or an acentric chromosome fragment (clastogenic phenomenon) that stood out of a chromosome after breakage [99].

In greater detail, in process of erythropoiesis, proliferating stem cells start to divide at which time a genotoxic agent may cause chromosome damage, such as breaks and exchanges. In addition, the genotoxic agent may also acts on macromolecules related to the function of chromatid disjunction, such as tubulin, causing spindle dysfunction (Figure 7). Consequently, a fragment or a whole chromosome may lag behind in the cell during division and may not become integrated into daughter nuclei, forming MN in the cytoplasm.

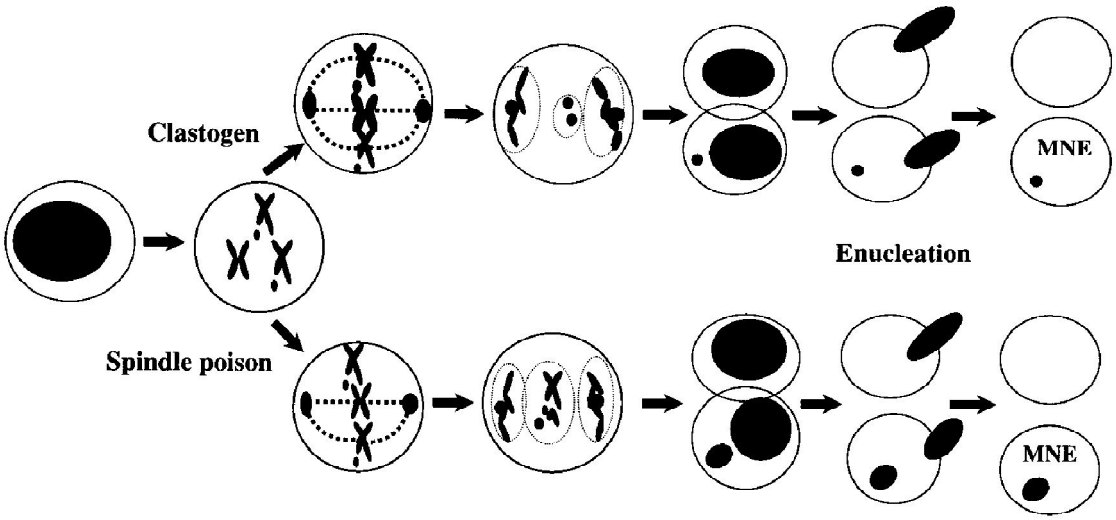

Figure 27. Scheme of the formation of micronucleated erythrocytes by mutagenic agents. MNE: Micronucleated erythrocytes [96].

Afterwards, during maturation in the process of erythropoiesis, the erythroblast develops into a polychromatic erythrocyte (PCE) that is a young erythrocyte still contains RNA, and the main nucleus is extruded. Therefore, if any MN has been formed during proliferation, it will remain in the enucleated cytoplasm and its visualization is facilitated because PCEs lack a main nucleus. PCEs, with time, lose RNA and develop into a normochromatic erythrocytes (NCEs), that are mature erythrocytes and they may also contain MN [97].

Traditionally, MN are identified using Giemsa staining method [99] but Hayashi *et al*. (1990) introduced a new method using acridine orange (AO) to improve the identification of immature erythrocytes [100]. PCEs are identified by red fluorescing reticulum in the cytoplasm, and MN fluoresce greenish yellow while NCEs fluoresce as pale green. AO staining method is more useful and gives more reliable results than the usual Giemsa because both PCEs and MN can be easily distinguished from NCEs [96].

MN represents an integrated response to chromosome-instability phenotypes and altered cellular viabilities caused by genetic defects and/or exogenous exposures to genotoxic agents. As a result, an increase in the frequency of MN is an indication of induced chromosome damage. Moreover, the PCE/NCE ratio between test agent-treated animals and vehicle-control animals provides a cytotoxicity index because a decrease in this ratio would indicate destruction of immature erythrocytes [97].

This assay has several important advantages over the analysis of bone-marrow metaphase analysis often used. It is technically simple, the endpoint scored is more objective and amenable for automation, it is less time consuming and it can be easily integrated into general toxicology studies [97]. This assay can be also applied to peripheral blood or other tissues or organs and it is a promising assay system, especially when used with FISH. With FISH, the presence of centromeres in MN can be clearly detected, and the ability to detect differences between MN induced by clastogens or by spindle poisons became possible [101, 102].

1.3.1.3. Sperm Abnormality Assay

There are several studies showing that pesticides have a direct effect on reproductive health, particularly in the formation of sperm cells [103-108]. The widely and first publicized report about these adverse effects was the case of pesticide dibromochloropropane (DBCP), in 1977, when Whorton *et al*. observed that male workers of a DBCP-producing factory in California in the United States became infertile after being exposed [109].

The DBCP story served to alert people about the potential for pesticides in to alter reproductive function in men. It also provided the rationale for adding direct evaluations of sperm production and quality to reproductive toxicology test protocols in which the rat is the preferred test species. The inclusion of measures of rat sperm quality, such as motility and morphology, into reproductive test protocols often increases the sensitivity of the test to detect effects [110].

Particularly, about morphological analysis, sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagens [111, 112]. This biomarker is advantageous because the reproducibility of the results and, overall the possibility to get information on the transmission of genetic damages in successive generations. According to Wyrobek and Bruce [1975], the classification of anomalies of the sperm is based on changes in the head and tail that is shown in Figure 8 [113].

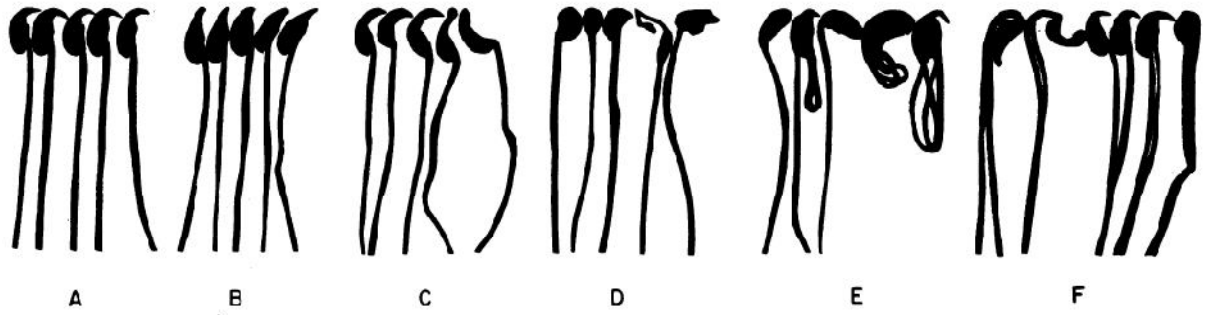

Figure 28. Classification of abnormalities in rodent sperm. A- Normal shape; B- Lack the usual hook; B- Banana-like form; D- Amorphous; E- Folded on themselves; F- Two tails [113].

Characteristics controlling sperm head shape are carried on the autosomes and it is not clear what mechanism origins these morphological changes. However, induction of abnormal sperms is presumed to be a result of naturally occurring errors in the differentiation process, or the consequence of an abnormal chromosome complement. In accordance to some authors the alterations in sperm can, in fact, occur as a consequence of DNA mutations and additionally by endocrine dysfunction [114-117].

1.3.2. Acetylcholinesterase Inhibition

Acetylcholinesterase (AChE) activity is widely used as a specific biomarker of organophosphorus (OPs) and carbamate insecticides that are commonly used in agriculture [118]. These anticholinesterase pesticides have the ability to inhibit AChE activity that is an enzyme responsible for modulating neural communication in the synaptic cleft by hydrolyzing the neurotransmitter acetylcholine. OPs and carbamates lead to a phosphorilation and carbamylation of the serine residue at AChE catalytic site, respectively. In that conditions, enzyme is highly stable and the hydrolysis of acetylcholine is blocked [119].The neurotransmitter accumulates in the synaptic cleft which originates the continuous stimulation of cholinergic fibers throughout the nervous systems. Depending on the degree of inhibition, cholinergic stimulation may lead to hyperactivity of excitable tissues, causing convulsions, severe muscle paralysis, hypersecretion from secretory glands, respiratory failure, coma and even death. The enzyme can be restored to its active state but in some cases, depending on the chemical structure of the pesticide, the inhibition may be irreversible [119, 120]. The Food and Agriculture Organization recommends that 20% inhibition is the relevant end-point to determine acceptable daily intakes of an anticholinesterasic compound [121].

Beyond this principal AChE involved in nerve impulse transmission, there are AChEs present in the outer membrane of erythrocytes and pseudocholinesterases present in blood plasma, liver and muscle tissues but their physiological function are unknown and it has a different substrate. Pseudocholinesterases are often inhibited more effectively by OPs and carbamates but their inhibition has no relation to that occurring in either erythrocytes or brain [122, 123]. The AChE activity in the brain is closely correlated with that measured in erythrocytes and to a lesser extent with pseudocholinesterase activity in the others tissues [76]. Notwithstanding, it is difficult to know how closely AChE inhibition in erythrocytes reflects that in the nervous system. Since access to blood is always easier than access to the brain, the inhibition of erythrocyte AChE is very useful as biomarker of exposure to pesticides and it usually overestimates AChE in the brain [123].

There are strong associations between exposures to pesticides and AChE inhibition symptoms. In accordance to some authors AChE is, in fact, significantly reduced in exposed populations [124-126].

Undoubtedly, the most widely used assay to detect AChE inhibition is the Ellman´s method. This method is extremely sensitive and is applicable to either small amounts of tissue and to low concentrations of enzyme [127]. This method is based on coupling of reactions presented in Figure 9:

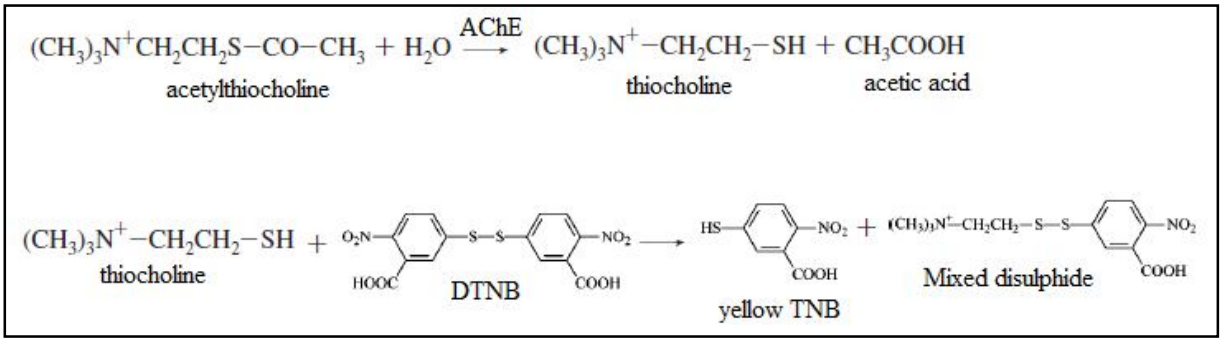

Figure 29. Principle of the Ellman´s method. AChE- acetylcholinesterase; DTNB- dithiobisnitrobenzoate ion; TNB- 5-thio-2-nitrobenzoate anion (adapted from[127]).

Therefore, the enzyme activity is measured by the increase of yellow -5-thio-2-nitrobenzoate anion (TNB) produced from the reaction of thiocholine with dithiobisnitrobenzoate ion (DTNB). Yellow TNB is quantified at 412 nm using a spectrophotometer.

The main advantages of the Ellman´s method are simplicity, accuracy, a continuous increase in color density as a function of incubation time, and relatively low cost. It is easily adaptable for automated analyzers or plate readers for the rapid processing of large numbers of samples [127].

.

1.3.3. Manganese Quantification as a Mancozeb Biomarker

Manganese (Mn) is an essential element that is required for growth, development, and maintenance of health. Within its multiple functions, it is a component and an activator of multiple important enzymes, like pyruvate carboxylase, superoxide dismutase or alkaline phosphatase and it is required for the formation of healthy cartilage and bone. Moreover, Mn is essential in the maintenance of mitochondria, production of glucose and in the urea cycle. It also plays a key role in wound-healing [128].

Mn is the 12th most abundant element in the Earth's crust and constituting about 0.1% of it. Pure Mn is a silver-colored metal but it does not occur as a free metal. Instead, it is found combined with oxygen, sulfur, and chlorine in more than 100 minerals and is one of the most used metals in the industry with a wide range of applications. Magnetic resonance imaging, antiknock agent and fungicides formulations are the most recent applications of this metal. Particularly, Mn enters in the constitution of Mancozeb, a fungicide extremely used in agriculture, at the present time [128].

The general population is exposed to Mn through consumption of food and water, inhalation of air, and dermal contact with air, water, soil, and consumer products that contain Mn. The primary source of Mn intake is through diet and Mn is naturally present in food and in drinking water at low levels. Daily intakes of Mn range from 2-9 mg/day depending on nutritional habits and only 3-5% is absorbed from the gastrointestinal tract [128]. However it is considered that the diet of most people in developed countries contains sufficient manganese [129]. The oxidation states of manganese range from 0 to +7 but the most stable valence is +2 and this is the state of Mn absorved from the gastrointestinal tract like tetravalent state [130]. This low gastrointestinal absorption together with a rapid elimination limit the toxicity of the Mn [130].

Despite its essentiality, possible involvement of high levels of Mn compounds in causing hazard effects on human health has been alert public to its utilization. On the other hand, Mn plays an important role in antioxidant defenses and forms part of a superoxide dismutase which often is characteristically modified in cancer cells. So, there is needed to consider the balance between essentiality and toxicity, when conduct of risk assessments for an essential element such as Mn [129].

It is consensual that chronic exposure to high levels of Mn is associated with adverse outcomes. John Couper, in 1837, was the first to report these Mn effects, when he described muscle weakness, limb tremor, whispering speech, salivation, and a bent posture in five men working in a Mn ore crushing plant in France [131]. This is the first description of manganism symptoms, a permanent neurological disorder caused by continued exposure to high levels of Mn. In fact, Mn has been shown to cross the blood-brain barrier, enters in the nervous system that is the primary target organ, originating behavioral changes and movements that may become slow and clumsy [130, 132, 133].

Despite the mechanism of toxicity of Mn is poorly understood, several studies indicate that oxidative stress and mitochondria play major roles in the Mn-induced neurodegenerative diseases [134, 135]. Moreover, there are some evidences that Manganese interferes with various mechanisms in nervous system, particularly in AChE activity. About interference of Mn in AChE activity, there have been conflicting reports regarding the effects of manganese treatment in developing rats. There are studies that indicate a significant inhibition AChE activity observed following lengthy periods of exposure to manganese [136]. On the other hand, Liapi *et al* (2008) suggests that short-term Mn administration causes a statistically significant increase in AChE activity in rats [137].

Mn appears to exert different effects on AChE activity in different stages of the life cycle. As a result, AChE activity is unaffected by manganese during development, but increased in a long-term exposure to manganese throughout development until adulthood. During aging, the physiological decrease in AChE activity is abolished by manganese [136, 138-141].

Effects of Mn at neurological level [142] are, in fact, the most investigated but Mn have also another target organs in respiratory, reproductive, endocrine and hematological systems [142-144]. Furthermore, a limited amount of manganese is also able to cross the placenta during pregnancy, thus potentiate its teratogenic action [128]. Information on cancer due to Mn is scanty but the results available do not indicate that Mn is carcinogenic. However, Mn is a proven mutagen, causing DNA damage and chromosome aberrations [129].

As previous mentioned, Mn enters on Mancozeb formulation. So, quantification of this metal in urine or in the organism can be used as a biomarker of exposure to Mancozeb. The most common analytical procedures for measuring Mn

levels in biological and environmental samples are the methods of Atomic Absorption Spectroscopy (AAS). Particularly, the Graphite Furnace Atomic Absorption Spectrometry (GFAAS) analysis is often used for very low analyte levels and for the analysis of solid samples [145]. In this method, the sample is aspirated into a graphite furnace until the element atomizes. Then, the ground-state atomic vapor absorbs monochromatic radiation from a source and a photoelectric detector measures the intensity of radiation absorbed. It is important to refer that this methodology requires acidic digestion of the organic matrix in the biological materials prior to analysis and that special care is needed to avoid contamination of biological materials with exogenous Mn, especially for samples with low levels of Mn [128, 146].

1.3.4. *CYP1A1* as a genetic marker of Susceptibility

Genetic polymorphism is a difference in DNA sequence among individuals, groups or populations that may have been induced by external agents. The simplest form and the most common source of genetic polymorphism in the human genome is Single Nucleotide Polymorphism (SNP). SNP is a single base mutation in DNA and is present at about 1 per 1,000 nucleotides in humans [147]. These polymorphisms can change the level of expression of a gene or change the activity of a gene product [148] which can be used a genetic marker of susceptibility when associated with an increased risk of develop certain diseases.

Through the recent advances in molecular biology, these genetic markers of susceptibility have been developing and now they play a key role in environmentally induced diseases. Individual inheritance of variant polymorphic genes involved in the metabolism of chemical compounds, such as pesticides, can be good markers of susceptibility because they allow to understanding the relationships between exposure to these toxic chemicals and the development of chronic diseases [32]. In more detail, genotypes responsible for inter individual differences in the ability to activate or detoxify pesticides will influence the genotoxic damage caused by this pesticides [73]. So, through the ability to sequence DNA and find individual genotypes, it is possible to discover the relationship between exposure and disease development according to genotype information. In case of humans, this is very useful because that information could be coupled with medical information to diagnose disease onset and to develop strategies of treatment [28].

As human population is biologically diverse and genetically heterogeneous, it is not surprising that differences in susceptibility to disease among individuals with or without exposure to environmental chemicals exist and many genetic conditions associated with enhanced susceptibility to environmental chemicals remain to be discovered [149]. There has been a great investment in this area and various genetic polymorphisms of enzymes involved in the metabolism of xenobiotics have been examined. As a result, some studies have reported an association between polymorphisms in cytochrome P450 (*CYP*) enzymes involved in human xenobiotics metabolism and susceptibility to cancer [150] .

Cytochromes P450 (*CYPs*) are a superfamily of 57 genes that encode enzymes involved in the typical activation reaction which converts indirect

carcinogens to active electrophiles capable of interacting with the biological macromolecules DNA, RNA and proteins [77, 151]. Particularly, the family of genes *CYP1A1*, *CYP1A2* and *CYP1B1* codify three enzymes, respectively, that detoxify or activate many procarcinogens, environmental toxins and some drugs. The activity of *CYP1A1* is induced by polycyclic aromatic hydrocarbons (PAHs), such as those found in cigarette smoke, diet and pollutants relapsed from the combustion of fossil fuels. Its gene product is a hydroxylase which catalyzes the first step of the metabolism of PAHs to electrophilic compounds. These enzymes are membrane associated proteins located in either the inner membranes of mitochondria and in the endoplasmic reticulum of cells, where they metabolize thousands of endogenous and exogenous compounds. The expression of *CYP1A1* is mainly extrahepatic. [152]. Many enzymatic isoforms of this enzyme have been suggested to contribute to individual cancer susceptibility as genetic modifiers of cancer risk after exposure to genotoxic agents [153, 154]. Moreover, some genetic polymorphisms of *CYP1A1* gene have been associated with an elevated risk of lung, colorectal and breast cancer [155-157].

The role of specific polymorphisms of *CYP* genes, involved in the activation and detoxification of xenobiotics, in modulating cytogenetic effects have been studied in pesticide-exposed populations [153, 154, 158]. Even though, the available data on farmer populations suggest that individuals with unfavorable metabolizing alleles are more susceptible to genotoxic effects than those with favorable alleles, but there are no conclusive findings on whether metabolic polymorphisms affect the chromosomal damage induced by pesticides.

1.4. Using Small Mammals as Bioindicators

The term bioindicator has been applied to living organisms whose characteristics are used to reveal the presence or absence of environmental conditions [159]. Thus, a bioindicator permits to evaluate a modification, usually a deterioration of the environmental quality and some wild species can carry out this purpose, being predictors of ecotoxicological consequences when contaminants are introduced in the environment [160].

Consequently, if animals living in polluted environments, like small mammals, accumulate toxicants with effects for their health, they can be used as bioindicators of that environmental pollution [161]. For example, morphological measures such as body and internal organs masses are commonly used to assess their health status [162, 163]. Moreover, the assessment of their hematological parameters also provides important information on their health and physiological status. Several studies have reported alterations of these measurements in small mammals when exposed to certain pollutants [162, 164]. Small mammals are able to accumulate a wide spectrum of pollutants which are present in the ecosystem [161] and, particularly, some significant correlations between pesticides and genetic damage in free-living rodents have been detected [165].

There are a lot of arguments for the use of small mammals in pollution biomonitoring and hazard assessment. They are often considered to represent an intermediate stage between low and high trophic levels, since they feed on herbs, fruits and invertebrates, and they constitute important items in the diet of carnivorous animals. Moreover, they participate actively in soil bioturbation and, despite their small body size, they have a high metabolic rate, being their degree of exposure greater than in large mammals, which have a slower metabolic rate [166-168].

On the other hand, the use of small mammals, mostly rodents in this type of work is very important because they have enough genetic and physiological similarities with humans and, therefore, it allows extrapolating the effect of these pollutants. Many rodent carcinogens are also human carcinogens, and hence, this model not only provides an insight into the genotoxicity of human carcinogens but also is suited for studying their underlying mechanisms.

The wild small mammal species, Algerian mouse (*Mus spretus* Lataste, 1883) and wood mouse (*Apodemus sylvaticus*, Linnaeus, 1758) were chosen in this study

as pesticide pollution bioindicators. These species have been widely used as a bioindicators in some environmental studies [160,162, 169-171].

Algerian mouse belongs to the family *Muridae* and it is a rodent species that inhabits the occidental portion of Mediterranean Europe and North Africa. They occupy sub-humid and semi-arid habitats, typically Mediterranean, and have an herbivore diet composed with seeds, leaves, stalks and fruits. Wood mouse also belongs to the family *Muridae* and it inhabits Europe and North Africa. They consume seeds, fruits, insect larvae, earthworms, or plant material depending on local and seasonal abundance [172, 173].

Figure 30. A- *Apodemus sylvaticus* [174]; B- *Mus spretus* [175].

These two species meet the criteria of good bioindicators because they are abundant and are easily caught; they contact with soil during their entire life cycle, being exposed to pesticides mainly by ingestion of contaminated food or soil as well through dermal absorption; their populations are usually large enough to support individuals collection without a major adverse effect at a population and they also are quite accessible for both population investigation and experimental research [176].

1.5. Thesis Context and Objectives

The widespread use of pesticides in agriculture represents a threat not only to the environment but also to human populations exposed to them. Conventional agriculture practices are largely responsible for the increased consumption of pesticides worldwide and, in Portugal, this consumption also have been increased. As a consequence, investments have been made in biological agriculture, without the use of pesticides, but this practice is still very restricted. Therefore, human exposure to pesticides continues to be inevitably and it is important to be alert in relation to their possible hazard effects.

The main aim of the present thesis is the *in situ* assessment of pesticides toxicity, using biomarkers to compare the health state of two small mammal species (*Mus spretus* and *Apodemus sylvaticus*) from conventional agriculture (CA) versus biological agriculture (BA) zone. Specific objectives of this thesis include:

1. to investigate the potential genotoxic effects of pesticides on small mammals, using Micronucleus, Comet and Sperm Abnormality assays as biomarkers;
2. to assess morphologic and physiologic conditions of both species;
3. to appraise the adequacy of acetylcholinesterase inhibition as a biomarker of exposure to organophosphates and carbamates;
4. to investigate the adequacy of manganese quantification as a biomarker of exposure to Mancozeb, the principal pesticide used in the studied CA zone;
5. to perform preliminary genotype analysis to try establish more frequent profiles for *CYP1A1* polymorphism gene that are involved in the activation and detoxification of xenobiotics like pesticides;
6. to appraise the adequacy of those biomarkers adopted;
7. to compare the two different species of small mammals from the two different zones to verify which one is the best bioindicator of pesticide exposure;
8. to evaluate the potential environmental risk upon wildlife as well as the likely impact on human health;

2. Material and Methods

2.1. Sampling and Animals Capture

This study was carried out in an agricultural zone of Alcobaça, district of Leiria, Portugal (Figure 11). In this region, agriculture is still an important economic activity with a wide area under agricultural practice.

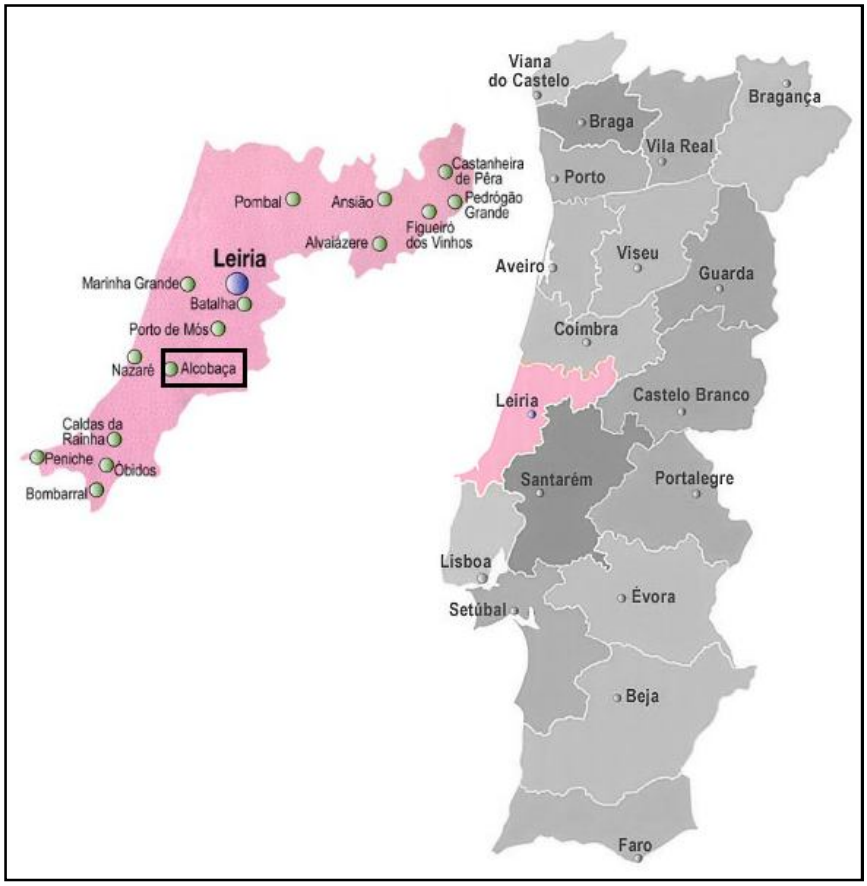

Figure 31. Geographic location of agricultural zones in study (adapted from [177]).

It was considered that conventional and biological agriculture zones (Figure 12) are both insert in the same biotope (≈1km of distance between them) and it was presumed that pesticide use is the main external factor that distinguishes them. The biological agriculture zone is correctly isolated from adjacent agriculture zones, so it is expected that it cannot be affected by adjacent pesticides applications.

Before animals capture, the agreement and the cooperation of the farmers involved were required and they were asked about pesticides and others chemical products that were used in the fields. While in biological agriculture (BA) zone, the owner farmer ensured that there were not used any type of chemical products, being

an agricultural practice based on the use of natural enemies for the pest control that was converted about 5 years ago, in conventional agriculture (CA) zone, the owner farmer provided a list of pesticides used, where OPs, carbamates insecticides and EBDCs were the most representative groups. Moreover, CA farmer ensure that more than 50% of the applications were based on one EBDC: Mancozeb. Thus, Mancozeb is the most used pesticide in the CA zone.

Figure 32. A- Conventional agriculture (CA) zone; B- Biological agriculture (BA) zone.

Approximately 60 small mammals of both sexes were captured between December 2010 and February 2011 in both zones (Figure 12). All the animals were treated according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [178]. Small mammals were captured with *Sherman* traps using a mixture of canned sardine, flour and oil as bait. Cotton was also placed inside each trap to minimize lower temperatures that were felt during the night. Pregnant or lactant females were discharged as well as animals with less than 10g of weight. Mice were housed in individual plastic cages until reach the laboratory with *ad libitum* access to food and water.

2.2. Animals Sacrifice and Morphophisiological Analysis

Small mammals were anesthetized and weighed before sacrifice and then general measurements were made (body size, tail size, left paw size, left ear size).

Cardiac puncture was done for blood collection, using a syringe with heparin to prevent coagulation and during dissection, organs were removed (liver, heart, kidneys, spleen, testis, femurs and brain) and weighed. Liver, heart, kidneys, spleen and brain were stored on cold (-70 ºC) for subsequent analysis.

Moreover, hematological parameters were taken from animal's blood using Beckman Coulter Act Diff Hematology Analyzer that measures the following parameters: WBC (white blood cells; $x10^3/\mu L$), RBC (red blood cells; $x10^6/\mu L$), Hgb (hemoglobin; g/dL), Hct (hematocrit; %), MCV (mean corpuscular volume; fL), MCH (mean corpuscular hemoglobin; pg) and MCHC (mean corpuscular hemoglobin concentration; g/dL).

2.3. Biomarkers of Genotoxicity

2.3.1. Comet Assay

The comet assay was performed as described by Singh *et al*. [86] with minor modifications. An aliquot of 15 µL of the blood samples collected by cardiac puncture was mixed with 300 µl 0.8% low-melting point agarose (37ºC) and then placed on a slide precoated with 1% normal-melting-point agarose. Immediately after that, the slide was covered with a coverslip and was then kept for 5 minutes on ice to solidify. After gently removing the coverslip, subsequent to solidification, slide was immersed in a cold lysing solution (2.5M NaCl, 0.1M EDTA, 10mM Tris, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) at 4ºC over night. After lysis, the slide was placed in a horizontal electrophoresis box. The box was filled with fresh electrophoresis solution (1 mM EDTA, 300 mM NaOH, pH≈13) and the slide was left in this solution for 40 min to allow unwinding. Electrophoresis was performed using the same solution at 25 V and 300 mA for 30 min. After that, the slide was gently neutralized with 0.4 M Tris buffer at pH 7.5 three times and then stained with SYBR Safe 4µL/mL. All steps described were performed under dark conditions to minimize additional ultraviolet induced DNA damage.

The slides were examined, and comet images were captured using a fluorescence microscope (Olympus BX41). Approximately 200 randomly selected cells were analyzed at 400x magnification per each animal. Extent of DNA migration was determined using a computerized image analysis system (Comet Score TM - TriTek Corp).

2.3.2. Micronucleus Assay

At the time of sacrifice, bone-marrow cells from both femurs of each animal were flushed with foetal calf serum, for the estimation of the frequency of micronucleated PCEs, according to Schmid [99]. The obtained cell suspension was centrifuged (800rpm, 5 min), the supernatant was removed and the pellet re-suspended in foetal calf serum. Then, a drop of the suspension was smeared on a clean slide, air-dried, fixed in methanol for 10 minutes and stained with AO for fluorescent microscopic examination (Olympus BX41, equipped with blue excitation and 515-530 nm barrier filter) with a magnification of 400×. The frequency of MN in 1000 PCE per mouse (coded slides) was scored. The acridine orange (0.24mM in Sorensen Buffer pH=6.8) used was prepared from a stock solution 0.1% AO according to Krishna and Hayashi (2000) [97].

2.3.3. Sperm Abnormality Assay

The testis of the mice was dissected out, placed in 1 mL of Sorensen buffer (pH 7.0) and gently centrifuged (800rpm, 10 min) to obtain a pellet of undamaged cells. After removal of the supernatant, the pellet was re-suspended in 1 mL of Sorensen buffer. A drop of the suspension was placed on a clean slide and a smear is made, air-dried and fixed in absolute methanol for 10 min. After drying overnight the slides were stained with 10% Giemsa for 1 h [179] and observed under the microscope with a magnification of 100×. According to Wyrobek and Bruce [113], 1000 sperm per animal were assessed for morphological abnormalities, which included without hook, banana shape, amorphous, folded on themselves and two tails.

2.4. Determination of Brain Acetylcholinesterase Activity

The brain acetylcholinesterase activity was measured spectrophotometrically according to Ellman *et al*. (1961) [180] in 10 *Mus spretus* and 10 *Apodemus sylvaticus* randomly selected from both zones (5 from CA zone and 5 from BA zone). Before enzyme reaction, 50 mg of each freeze-thawed brain sample were homogenized in 0.9 mL of 0.1M sodium phosphate buffer (pH 7.4) with 0.1mL of Triton X-100 5% (v/v), using a Potter homogenizer.

Then, for the enzymatic assay, 25 µL of the homogenized brain were added to the reaction mixture contained 50µL of DTNB (3.96mg/mL) and 1375 µL of 0.1M sodium phosphate buffer (pH 7.4). Subsequently, 50 µL of acetylthiocholine iodide (ACT) (9.248mg/ml) were added and the mixture was vortex for a few seconds. With the addition of ACT, the enzymatic reaction begins and the reading in the Beckam 640 spectrophotometer at 412 nm had to be immediate. All steps described were performed under cold conditions, on ice, to minimize enzymatic activity before the enzymatic reaction begins. Changes in absorvance were recorded for a period of 3 minutes at intervals of 10 seconds. This enzymatic assay was always done in triplicates and the enzymatic activity was expressed as µmol ACT hydrolyzed/min/g of brain and nmol ACT hydrolyzed/min/mg of protein.

The protein content of the homogenates was determined according to the method of Bradford [181] using bovine serum albumin as standard.

2.5. Determination of Manganese in the Liver

About 150 mg of each sample of liver pre-stored at -70ºC from 10 *Mus spretus* and 10 *Apodemus sylvaticus* randomly selected from both zones (5 from CA zone and 5 from BA zone) were defrosted and dried at 80ºC until reaching a constant weight. After that, dried samples were digested with 5 mL of 65% suprapure nitric acid during 3 hours at 150ºC.

The resulting clear acid solutions were transferred to 50 mL volumetric flasks that were completed with deionized water. These solutions were used for the determination of Mn concentrations that were determined by GFAAS with a PerkinElmer AAnalyst™ 700 atomic absorption spectrometer equipped with an HGA Graphite Furnace and a programmable sample dispenser (AS 800 Auto Sampler and

WinLab 32 for AA software). $Mg(NO_3)_2 \cdot 6\ H_2O$ (0.84 mol/L) was used as a chemical modifier. Samples were measured at least twice and every measurement consisted of two separate injections into the graphite furnace.

Results were expressed as micrograms of Mn per gram of dry liver. Calibration curves were automatically obtained by the device starting from a 25µg/L solution of $MnCl_2$ that was successively diluted for 12.5, 6.3, and 2.5 µg Mn/L. The limit of detection was 0.05 µg Mn/L.

2.6. Preliminary Genotype Analysis

For the genotype analysis of *CYP1A1* polymorphism gene, it was used Tissue DNA Kit (Omega bio-tek) for extraction of DNA in muscle tissues. After that, Polimerase Chain Reaction (PCR) was performed for amplify samples of DNA, using specific primers available on lab that flanking one region of *CYP1A1* gene with 702 bp (Figure 13). It was used 15.5 µL of water, 5 µL of buffer (1x), 1 µL of dNTPs (2.5 mM), 1.5 µL of magnesium (1.5 mM), 0.2 µL of primers F and R (25 µM), 0.4 µL of BSA (0.16 µg/µL), 0.2 µL of Taq polymerase (5U/µL) and 1 µL of DNA, for a total reaction volume of 25 µL per tube. Thermal cycling conditions included a hot start at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1min, annealing at 66ºC for 2 min and extension at 72ºC for 1 min. Final extension was performed at 72ºC for 5 min. Then, samples were subjected to electrophoresis in a 1% agarose gel. Moreover, two samples successful amplified was purified and then send for sequencing.

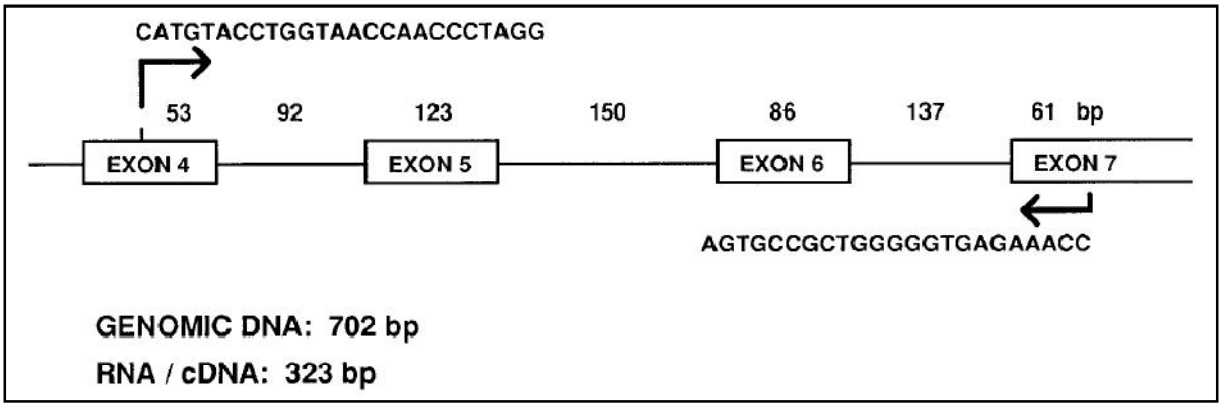

Figure 33. Diagram of exons (rectangles) and introns (lines) of the mouse *Cyp1A1* gene. The primers used for PCR analysis are illustrated, which are designed to produce a genomic DNA PCR fragment of 702 bp. Numbers above denote base pairs (bp) of the exons and introns [182].

In addition, successful amplified PCR products were digested during 3h at 37ºC with restriction enzymes MseI and EcoRI, whose recognition sites are TTAA and GAATTC respectively, in order to assess if there was a differential cut. Through the GenBank, it was possible to consult the sequence of the fragment amplified and predict the number of fragments originated by these enzymes: MseI should cut the fragment at two sites, forming three fragments and EcoRI should cut the fragment at one site, forming two fragments. It was used 1 µL of 10x buffer, 2µL of DNA, 1 µL of MseI or 0.5 µL of EcoRI and 6 or 6.5 µL of water, respectively for a total reaction tube volume of 10 µL. After digestion, samples were subjected to electrophoresis in a 1% agarose gel.

2.7. Statistical Analysis

The statistical analysis of the two species captured was done separately because of the differences between them. The data were statistically analyzed using SPSS v.19.0. All variables were checked for normal distributions, using Kolmogorov-Smirnov test.

Differences between conventional agriculture group and biological agriculture group were assessed with Student's *t*-test if they exhibited a normal distribution; if not, the Mann-Whitney`s U-Test was used instead. Differences between males and females of each zone were always assessed. The significance of differences was examined at the *p*-value of 0.05.

For alignment and sequence analysis were used BioEdit and Sequencher 4.8 programs.

3. Results

3.1. Morphophisiological Analysis

From the 60 small mammals captured in both zones, only 46 were analyzed because pregnant or lactant females were discharged as well as animals with less than 10g. The distribution and morphophisiological measurements are presented on Table 4.

Table 4. Distribution and means for body measurements, internal organs mass and hematological parameters of the small mammals captured.

	Mus spretus		***Apodemus sylvaticus***	
	CA zone	BA zone	CA zone	BA zone
Number of males	8	9	6	2
Number of females	6	5	3	7
Total number of animals	14	14	9	9
	Mean±SD			
Body measurements:				
Body Weight (g)	12.41±1.87	11.74±1.12	22.50±3.11	20.90±3.89
Body Length (cm)	13.22±0.92	12.48±0.39	17.80±1.25	17.35±1.33
Relative Length left paw (%)	11.62±0.43	11.58±0.68	11.96±0.71	12.06±0.62
Relative Length left ear (%)	9.31±0.57	8.96±0.58	9.02±1.28	8.42±0.65
Internal organs mass:				
Relative Weight spleen (%)	0.33±0.11*	0.24±0.08*	0.36±0.25	0.22±0.08
Relative Weight liver (%)	5.88±0.95	5.57±1.09	5.04±0.70*	6.32±1.01*
Relative Weight kidneys (%)	0.78±0.07	0.77±0.06	0.61±0.09	0.54±0.07
Relative Weight testis (%)	0.40±0.12*	0.61±0.07*	1.49±0.51	1.65±0.11
Relative Weight Brain	2.76±0.35	2.91±0.33	2.62±0.42	2.81±0.38
Hematological parameters:				
WBC ($x10^3/\mu L$)	7.94±4.85	5.92±2.18	9.36±12.31	5.27±1.51
RBC ($x10^6/\mu L$)	6.03±0.86	6.22±1.14	6.02±1.42	6.64±0.64
Hgb (g/dL)	12.75±0.99*	11.07±1.36*	11.53±1.72	11.98±0.98
Hct (%)	26.74±3.99	26.89±4.25	28.51±5.14	30.83±2.58
MCV (fL)	44.40±3.09	43.39±2.14	48.03±3.70	46.52±3.24
MCH (pg)	21.46±2.95**	18.02±1.48**	19.68±3.25	18.10±1.57
MCHC (g/dL)	48.35±5.71**	41.56±3.10**	40.84±4.75	38.94±2.42

*p<0.05 by Student`s T-Test; **p<0.05 by Mann-Whitney`s U-Test, significant differences between CA and BA zones (CA-Conventional agriculture, BA-Biological agriculture, SD-Standard deviation, WBC-White blood cells, RBC-Red blood cells, Hgb-Hemoglobin, Hct-Hematocrit, MCV-Mean corpuscular volume, MCH-Mean corpuscular hemoglobin, MCHC-Mean corpuscular hemoglobin concentration).

There were no significant differences for morphophisiological measurements between males and females of each zone, thus sexes were combined for statistical analysis. All measures were normally distributed except body length (D=0.236, p=0.034) and MCHC (D=0.352, p<0.001) for *Mus spretus* from CA zone, WBC (D=0.416, p<0.001) for *Apodemus sylvaticus* from CA zone and MCHC (D=0.294, p=0.024) in *Apodemus sylvaticus* from BA zone.

About Internal organs mass, significantly higher mean values of relative weight spleen (t=2.405, p=0.024) were found for *Mus spretus* from CA zone compared to BA zone. Moreover, *Mus spretus* from BA zone have a higher mean value of relative weight testis (t=-4.471, p=0.001). In relation to *Apodemus sylvaticus*, there were find significant differences only for the relative weight liver, where animals from BA zone have significantly bigger livers (t=-3.126, p=0.007).

In relation to hematological parameters, there were no significant differences between CA and BA groups of *Apodemus sylvaticus*. However, there were significant differences between *Mus spretus* from CA and BA zones for hemoglobin (t=3.735, p=0.001), mean corpuscular hemoglobin (t=3.905, p=0.001) and mean corpuscular hemoglobin concentration (U=13.00, p<0.001).

3.2. Biomarkers of Genotoxicity

3.2.1. Comet Assay

Extent of DNA migration was assessed using the parameter percentage of DNA in the comet tail (% tail DNA). This parameter of DNA damage is widely used and correlates well with visual scores. Figure 13 shows examples of visual aspect of comets from *Mus spretus* lymphocytes with different levels of DNA damage. Comets from *Apodemus sylvaticus* have a similar aspect.

About % tail DNA, there were no significant differences between males and females of each zone, thus sexes were combined for statistical analysis. % tail DNA was not normally distributed for *Mus spretus* (D=0.233, p=0.038) and *Apodemus sylvaticus* (D=0.286, p=0.033) samples from CA zone.

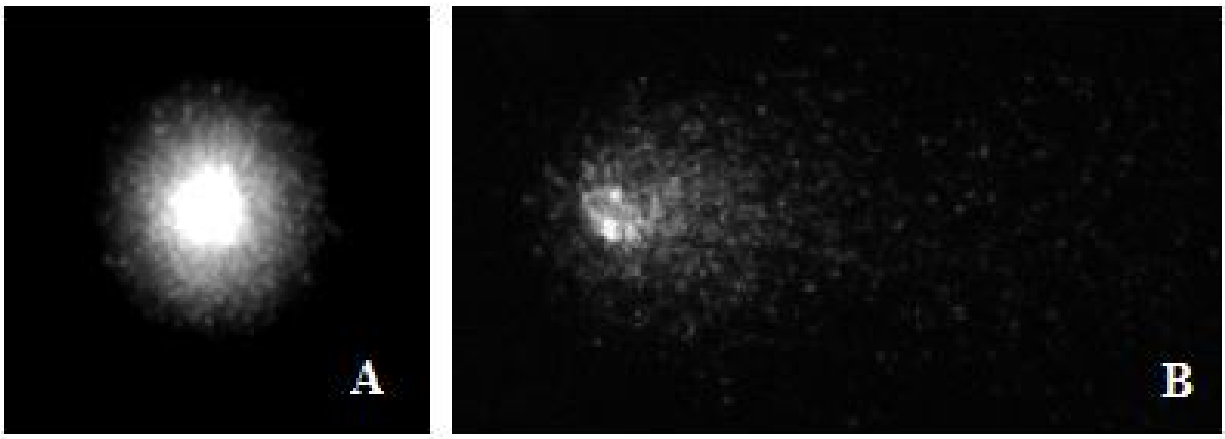

Figure 34. Examples of comets (from *Mus spretus* lymphocytes) stained with SYBR safe, showing different levels of DNA damage: A) Comet with low DNA migration that means few DNA damage and B) a comet with increased DNA migration (increased DNA damage) with a large tail.

As we can see in Figure 15, for both species there is a tendency for greater genetic damage in animals from CA zones, which are exposed to pesticides. However, there is a significant difference between CA zone (Mean % tail DNA=9.80±2.48) and BA zone (Mean % tail DNA=6.96±2.63) only for *Apodemus sylvaticus* (*U*=16.00, *p*=0.031). *Mus spretus* from CA zone (Mean % tail DNA=8.10±3.04) and *Mus spretus* from BA zone (Mean % tail DNA=6.96±2.63) do not show significant differences (*U*=85.00, *p*=0.550) relatively to the DNA damage estimated by % tail DNA.

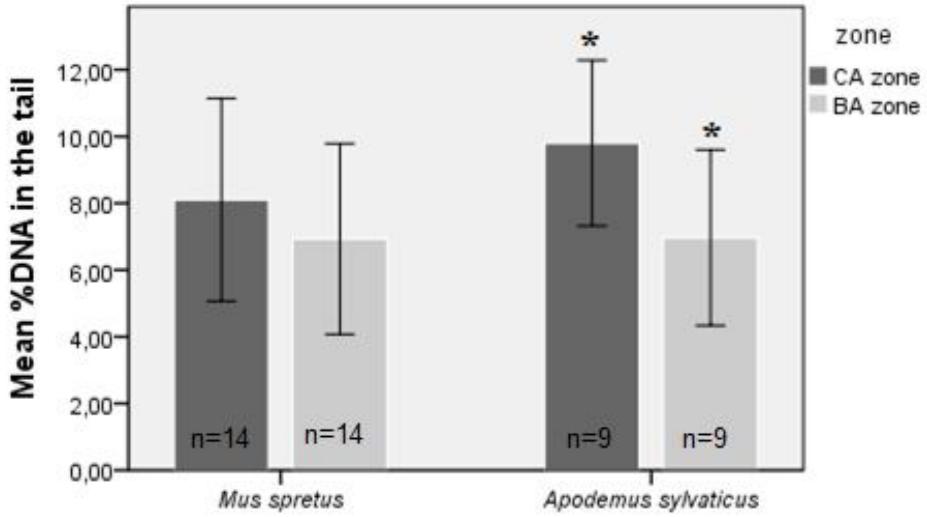

Figure 35. DNA damage assessed by mean of % DNA in the tail of the lymphocyte comets from small mammals of the different groups analyzed. Error bars represent the standard deviation of the mean (*p<0.05 by Mann-Whitney`s U-Test, significant differences between CA and BA zones; CA zone-Conventional agriculture zone; BA zone-Biological agriculture zone; n= number of tested animals).

3.2.2. Micronucleus Assay

With Acridine Orange staining, cytoplasm of PCEs emits red fluorescence and MN fluoresces yellowish green. NCEs emit green fluorescence and they were easily distinguished from PCEs (Figure 16).

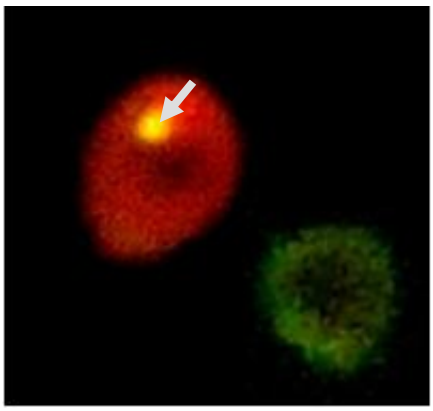

Figure 36. Acridine Orange stained bone-marrow erythrocytes of *Mus spretus*. PCEs are identified by red fluorescing reticulum in the cytoplasm and MN fluoresced greenish yellow while NCEs fluoresced as pale green. Arrow indicates a micronucleus (MN) in a polychromatic erythrocyte (PCE).

There were no significant differences for MNPCEs number between males and females of each zone, thus sexes were combined for statistical analysis. MNPCEs number was not normally distributed for *Mus spretus* from CA (D=0.252, p=0.0316) and BA (D=0.227, p=0.048) zones.

As we can see in Table 5, for both species there is a tendency for greater genetic damage in animals from CA zones, which are exposed to pesticides. However, there is a significant difference between CA (Mean % tail DNA=9.80±2.48) and BA zones (Mean % tail DNA=6.96±2.63) only for *Mus spretus* (U=14.00, p<0.001). *Apodemus sylvaticus* do not show significant differences (t=1.482, p=0.158) relatively to the DNA damage estimated by MNPCEs number, between the two zones.

Table 5. Frequency of micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes from bone-marrow cells.

	Mean ± SD			
	Mus spretus		***Apodemus sylvaticus***	
	CA zone n=14	BA zone n=14	Ca zone n=9	BA zone n=9
number of MNPCEs	6.21±2.26*	2.07±2.17*	3.22±1.56	2.11±1.62

***p<0.05 by Mann-Whitney`s U-Test; significant differences between BA and CA zones (BA-Biological agriculture; CA-Conventional agriculture; SD-Standard deviation; MNPCE-Micronucleated polychromatic erythrocytes; n= Number of tested animals).

3.2.3. Sperm Abnormality Assay

In this assay different morphological alterations in the mice sperm were observed (Figure 17). Amorphous sperm and sperm without the usual hook are the most frequently observed alterations. On the contrary, the two tails and the banana form are the least common morphological abnormalities (Table 6).

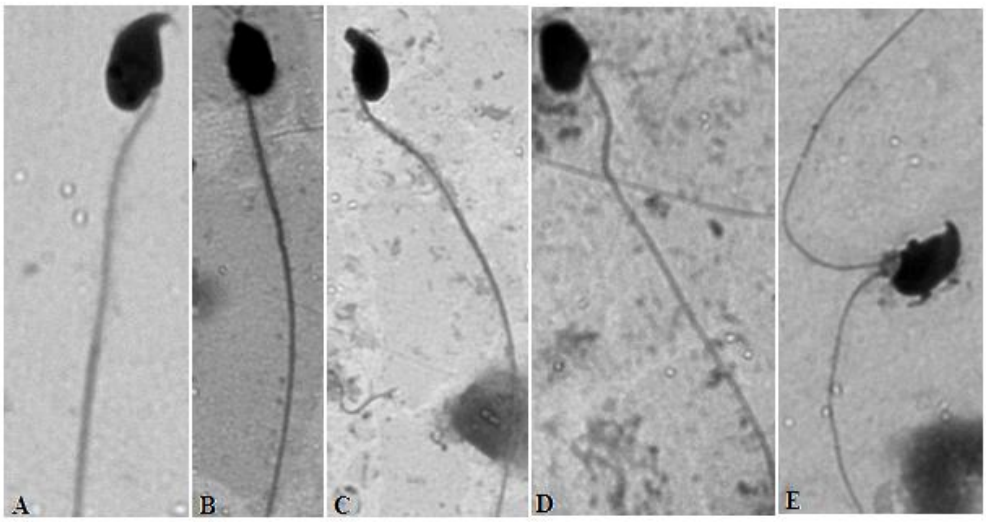

Figure 37. Different morphological alterations in mice sperm. A- Normal shape; B- Lack the usual hook; B- Banana-like form; D- Amorphous; E- Two tails.

Abnormal sperm frequency was normally distributed for all the samples and it increased significantly in *Mus spretus* from CA zone when compared with *Mus spretus* from BA zone (t=3.201, p=0.006). So, small mammals exposed to pesticides have increased abnormal sperm. *Apodemus sylvaticus* from CA zone also present a higher frequency of abnormal sperm compared to BA zone but this result is not statistically significant (t=0.500, p=0.638).

Table 6. Frequency of different types of sperm abnormality and the total abnormal sperm.

	Mean ± SD			
	Mus spretus		***Apodemus sylvaticus***	
	CA zone n=8	BA zone n=9	Ca zone n=6	BA zone n=2
Hook loss (%)	3.20±1.29	1.46±0.81	1.12±0.67	0.50±0.00
Banana (%)	0.96±0.59	0.58±0.40	0	0
Amorphous (%)	2.71±2.24	1.00±0.50	0.94±0.36	0.90±0.42
Two tails (%)	0.025±0.046	0.03±0.1	0.10±0.10	0.35±0.21
Total abnormal sperm (%)	6,9±3.29*	3.07±1.39*	2.16±1.09	1.75±0.21

*****p<0.05 by Student`s T-Test; significant differences between BA and CA zones (BA- biological agriculture; CA- conventional agriculture; SD-standard deviation).

3.3. Determination of Brain Acetylcholinesterase Activity

Brain AChE activity was expressed not only as µmol ACT hydrolyzed/min/g of brain but also as nmol ACT hydrolyzed/min/mg of protein, being protein content determined according to the method of Bradford. AChE activity expressed as nmol ACT hydrolyzed/min/mg of protein was not normally distributed for *Apodemus sylvaticus* (*D*=0.385, *p*=0.002) from BA zone.

For both species, there is a tendency for greater activity of brain AChE in the CA zone, exposed to pesticides (Table 7). However, the differences between CA and BA zones are not statistically significant (*Mus spretus*: [1]*t*=1.207, *p*=0.262; [2]*t*=0.991, *p*=0.351; *Apodemus sylvaticus* [1]*U*=11.000, *p*=0.291; [2]*U*=4.000, *p*=0.076).

Table 7. Brain acetylcholinesterase activity.

	Mean ± SD			
	Mus spretus		***Apodemus sylvaticus***	
	CA zone n=5	BA zone n=5	Ca zone n=5	BA zone n=5
ACh activity				
µmol ACT hydrolyzed/ min/g brain	15.11±4.91	12.13±2.49	11.95±3.49	9.63±2.60
nmol ACT hydrolyzed/ min/mg protein	0.0027±0.0010	0.0022±0.0005	0.0022±0.0006	0.0018±0.0004

*p<0.05 by Student`s T-Test; **p<0,05 by Mann-Whitney`s U-Test, significant differences between CA and BA zones (CA-Conventional agriculture, BA-Biological agriculture, SD-Standard deviation).

[1] AchE activity expressed as µmol ACT hidrolized/min/g of brain;

[2] AchE activity expressed as nmol ACT hidrolized/min/g of protein.

3.4. Determination of Manganese in the Liver

Mn concentrations in the liver were normally distribuited for all the samples. Both *Mus spretus* and *Apodemus sylvaticus* from CA zone had statistically significant high levels of Mn in the liver compared to animals from BA zone (Figure 18).

For *Mus spretus*, animals from CA zone had 7.20±1.87 µg Mn/g dry liver, while animals from BA zone had 4.21±1.09 µg Mn/g dry liver (t=3.078 p=0.015). For *Apodemus sylvatcus*, animals from CA zone had 7.65±1.12 µg Mn/g dry liver, while animals from BA zone had 4.85±1.83 µg Mn/g dry liver (t=2.915 p=0.019).

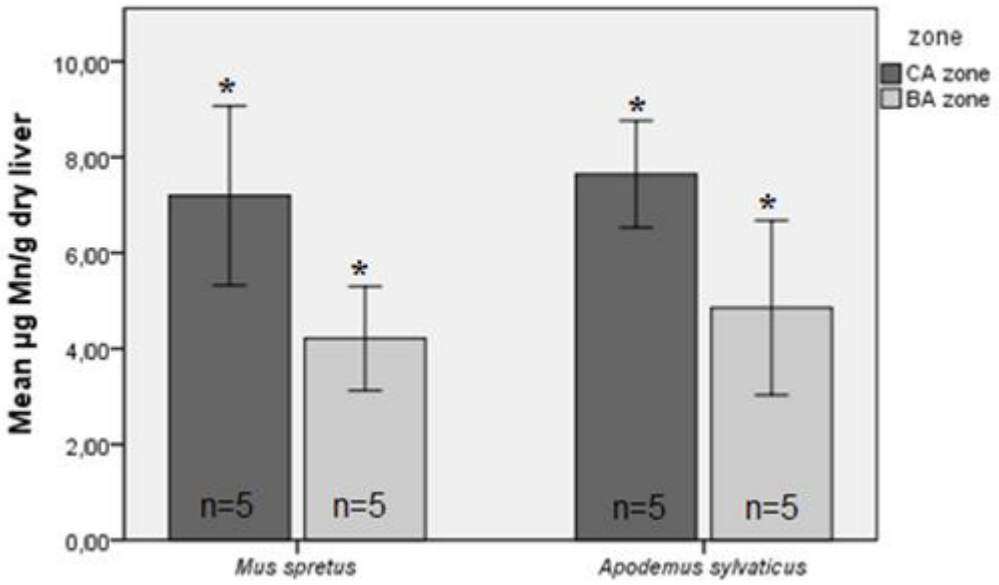

Figure 38. Mn quantification from small mammal´s livers of the different groups analyzed. Error bars represent the standard deviation of the mean (*p<0.05 by Student´s T-Test, significant differences between CA and BA zones; CA zone-Conventional agriculture zone; BA zone-Biological agriculture zone; n= number of tested animals).

3.5. Preliminary Genotype Analysis

The electrophoresis result of 10 *Mus spretus* muscle samples successful amplified with PCR are presented in Figure 19. As we can see, all samples were correctly amplified because there is a single band (≈ 700bp) of DNA for each sample. For *Apodemus sylvaticus,* there were not successful amplified samples with the same primers used for *Mus spretus*.

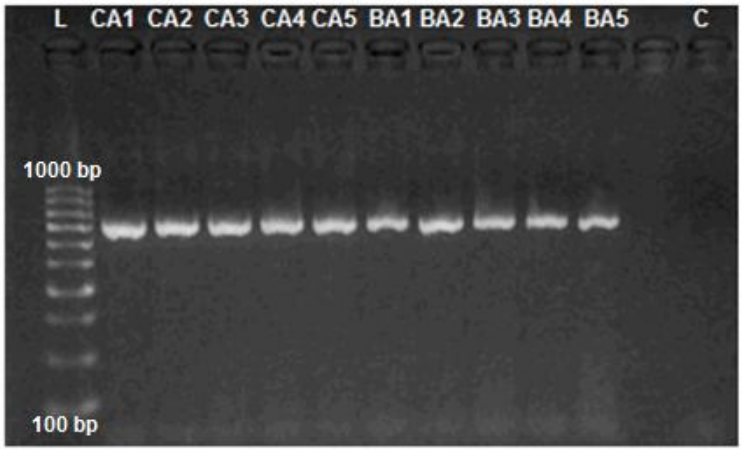

Figure 39. Photograph of agarose gel subjected to electrophoresis after PCR reaction. There were analyzed 5 *Mus spretus* samples from CA zone (CA1, CA2, CA3, CA4, CA5) and 5 *Mus spretus* from BA zone (BA1, BA2, BA3, BA4, BA5). L represents the leader and C is a control assay.

After analysis of the two samples sequenced, it was found only one different base pair on the fragment amplified between the two animals.

About enzymatic digestion, MseI and EcoRI originate the 3 and 2 fragments expected, respectively and there was no differential cut, when comparing CA and BA samples (Figure 20).

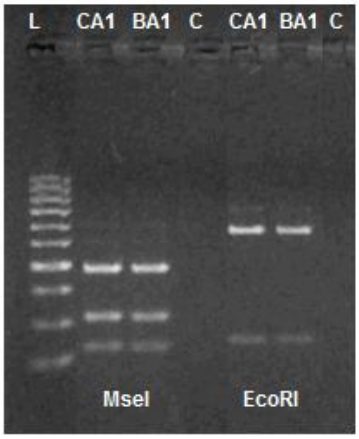

Figure 40. Photograph of agarose gel with PCR samples subjected to enzymatic digestion. CA1 is a sample from CA zone while BA1 is a sample from BA zone. L represents the leader and C is a control assay for each enzymatic digestion.

4. Discussion of Results

Pesticides are widely used in modern agriculture despite their hazards to humans and to nature. They have played a key role in providing reliable agricultural products at prices affordable to consumers, improving the quality of produce, and ensuring high profits to farmers. Theoretically, they are developed to function with reasonable certainty and minimal impact on human health and the environment. At European level, according to 91/414/EEC Directive (Council Directive 91/414/EEC, 1991), active substances in pesticides formulations are only approved for use if they have undergone a peer-reviewed safety assessment [19]. Despite of that, serious concerns have been raised about health risks resulting from exposure to these approved pesticides and numerous studies have been published in order to alert about this [7,31, 183].

In particular, the low reported acute toxicity and short environmental persistence of Mancozeb have allowed its worldwide usage in agriculture, even though numerous effects of its chronic exposure have been reported. In fact, as stated before, Mancozeb has the capability to alter the function of thyroid [58, 59], immune system [60], reproductive system [61-64] and nervous system [65]. Moreover, in a long-term experimental study, Belpoggi *et al.* (2002) verified that Mancozeb is a multipotent carcinogen inducing a variety of tumors of different origins in rats [66]. Nevertheless, Mancozeb is included in the list (referred to as Annex I of the Council Directive 91/414/EEC, 1991) of active substances that are considered acceptable in regard to their impact on the environment, human and animal health, and therefore authorized for use in pesticide formulations within the European Community [19].

The present study clearly demonstrates that exposure to a mixture of pesticide, where Mancozeb represents more than 50% of that mixture, has the ability to cause DNA damage in small mammals, assessed by comet, MN and sperm abnormality assays. These results are in accordance with other studies using comet assay [67], MN assay [184] as well as the sperm abnormality assay [185] to assess Mancozeb genotoxicity.

In a situation of chronic exposure to a mixture of pesticides like that, it is difficult to make sure that genotoxic effects detected are due to a particular pesticide. The owner farmer from CA zone ensures that more than 50% of the pesticide

applications were based on Mancozeb, an EBDC fungicide, but some OPs and carbamates insecticides were also used. So, to verify if the genetic damage detected would be due to this insecticides action, AChE activity was determined. AChE activity is widely used as a specific biomarker of these pesticides that are commonly used in agriculture [118] and if the small mammals analyzed were subjected to their action, AChE activity will be inhibited. In the present study, there were no significant differences between AChE activity for small animals from CA and BA zones that could explain the significant differences of the genetic damage found in the same animals. Thus, we can conclude that OPs or carbamates insecticides are not the genotoxic agents at issue.

On the other hand, to prove that Mancozeb, the most widely used pesticide in the CA zone, was the genotoxic agent responsible to the DNA damage detected, it would be necessary to prove that these animals were really exposed. Mancozeb is an EBDC with the presence of coordinated transitional metals and ETU is the specific metabolite of these compounds resulting from a spontaneous breakdown. Therefore, ETU quantification in urine has been widely used as a biomarker of exposure to Mancozeb and others EBDCs[76]. However, in this study, it was not possible to use this biomarker to assess exposure to Mancozeb because the amounts of urine obtained from small mammals were not sufficient to quantify ETU. To achieve this, it would be necessary to keep animals in metabolic cages for several days, which was not possible.

Alternatively, it was used Mn quantification by GFAAS to indirectly quantify Mancozeb inside the animal's body. Mancozeb is chemically characterized by the presence of coordinated transitional metals in the molecule, like Mn and Zn. Thus, measurement of these metals in biological tissues has been proposed as an alternative approach to monitor Mancozeb exposure. This application may be very useful for biological monitoring, since determination of metals can be performed with very sensitive AAS methods which are of lower complexity than those required for metabolite determination, like ETU [52]. Canossa *et al*. (1993) have already observed an increased urinary excretion of manganese in workers exposed to Mancozeb [186] but more research is needed because the available data, at present, are not sufficient to validate the use of metals as biomarkers of exposure to Mancozeb [52]. In addition, it is necessary to investigate the presence of metals not only in urine but also in other tissues and biological fluids as potential biomarkers.

Consequently, in the present study, it was used Mn quantification by GFAAS in small mammals' liver as a potential biomarker of Mancozeb exposure, because the liver is a filter organ where metals will be accumulated. As a result, it was found that both species (*Mus spretus* and *Apodemus sylvaticus*) from CA zone, exposed to pesticides, with greater genetic damage, have also greater levels of the metal Mn, comparing to animals from BA zone. So, considering that Mancozeb is the widely used pesticide in the CA zone, we can conclude that this pesticide will be the source of the highest levels of Mn found in small mammals. In other words, it seems that Mancozeb is the genotoxic agent which caused the DNA damage detected.

It is still important to note that, although results are not significant, there is a tendency for higher values of AChE activity in the CA zone compared to BA zone, which is not in line with expectations because CA zone is exposed to pesticides, including OPs and carbamates insecticides, which supposedly will inhibit the enzyme. However, it is known that AchE activity has to be determinated immediately after exposure and during captures there were no pesticides applications. So, AchE activity is not a good biomarker when the objective is to evaluate the long-term exposure. On the other hand, the presence of higher levels of Mn, as a consequence of the high amounts of Mancozeb used, can be related to these higher levels of AChE activity. In fact, Liapi *et al*. (2008) has recently suggests, in a short-term Mn administration study, that Mn cause a significant increase in AChE activity in rats [137] and our results seem to be in concordance with these ones. Although Mn action is a complex process and is still very controversial, Mn exerts different effects on AChE activity in different stages of the life cycle [138].

Despite the evident genotoxic action of Mancozeb, the two analyzed species seems to be affected in a different away. While, for *Mus spretus* significant genetic differences were detected by the MN and sperm abnormality assays, for *Apodemus sylvaticus* significant genetic differences between animals from CA and BA were only detected by comet assay. Comet, MN and sperm abnormality assays assess the DNA damage in different cells populations: comet assay evaluate the DNA damage in lymphocytes from the bloodstream, MN were detected in PCEs from bone marrow and sperm abnormalities were assessed in male germ cells. Thus, our results suggest that the two species subject to the same genotoxic agents appear to have populations of cells to be affected differently.

The differential response to the same genotoxic agent may be due because these two species have very different habits of life, including different feeding habits. *Mus spretus* are exclusively herbivores, whereas *Apodemus sylvaticus* are more omnivorous and their diets may include a large fraction of arthropods. Thus, the intensity and the frequency of the animal`s exposure to pesticides will be different in the two species because different foods will have different levels of these chemicals. On the other hand, different responses to genotoxic agents may only reflect intrinsic differences of species rather than differences related to exposure that was already corroborated in previous studies [187, 188].

It would be expected that Comet assay was a more sensitive test than MN assay because there are a lot of studies that confirm the ability of comet assay to detect the effect of genotoxic agents at lower doses than the MN assay. However, taking into account the present study, it seems that this sensibility depend on the species concerned. For *Mus spretus*, MN assays was the ability to detect significant differences in DNA damage between small mammals from CA and BA zones that were not detected by Comet Assay. MN assay using AO as staining method is more useful and gives more reliable results than the usual Giemsa staining because both PCEs and a MN can be easily distinguished. So, MN assay using AO staining has also a good sensibility that should be considered.

On the other hand, the sensitivity difference between the comet assay and MN assay may be due to their different endpoints. MN assay determines unrepaired DNA strand breaks, while the comet assay determines strand breaks (single and/ or double) and labile sites that are subsequently removed by repair enzymes [189]. Comet assay can determine the short-lived DNA damage while the MN assay detects the structural and numerical chromosomal damage [97, 190]. Thus, like several reports suggests, a combination of this two assays might therefore be recommended to understand the mechanisms underlying mutagenicity and to improve the sensibility as well as reliability of detection which can better facilitate the risk assessment process [191].

Furthermore, a combination of these two assays with the sperm abnormality assay is also very useful because it is a fast and sensitive mutagenicity test [113] used to detect cytogenetic damage in germ cells further than somatic cells. However, the classification used in this test is somewhat subjective with many technical artifacts and, therefore, it has not been widely used in recent times. For example, in

the present study, we have to discard the category “folded on themselves” of the sperm abnormalities classification because there was found an abnormal percentage of sperm cells in this category for all the animals due possibly to a very high centrifugal speed. Nevertheless, we find significant differences in the percentage of sperm abnormalities in *Mus spretus* from CA zone, comparing to BA zone, but there were no significant differences for *Apodemus sylvaticus*. It is important to refer that this result can be due to the reduced number of males *Apodemus sylvaticus* captured: only six males in CA zone and two males in BA zone. In fact, a larger sampler would be essentially for more reliable results not only for sperm abnormality assay but also for the other genotoxic tests, especially in wild species like these because the variability among animals can be higher than in laboratory animals.

Our study clearly showed that the use of only one cytogenetic end point is not sufficient to understand the range of the effects of pesticides on the genetic material in an *in situ* study. However, when other end points are included like in the present study, the method can be very promising. Moreover, because of differences in sensitivities, it is important to have more than one species for comparison to establish a genotoxic profile of one area.

About morphophisiological analysis, it will be expected that animals from contaminated zone (CA zone) would have lower values for body measurements comparing to uncontaminated zone (BA zone). In fact, there have been recognized morphological changes in organisms as a result of environmental pollution and body measurements, internal organs mass as well as hematological parameters are commonly used to assess the health status of wild species exposed to any kind of pollution [162, 163]. So, it will be expected that exposure to pesticides had influence on animals development and consequently on animal size. However, there were no significant differences for body measurements between animals from CA and BA zones for both species. Significant differences were only found in some internal organs mass: *Mus spretus* from CA zone have significant lower values of relative weight testis comparing to animals from BA zone. Moreover, significant differences were also found in relative weight spleen for *Mus spretus*, where animals from CA zone have bigger spleens than animals from BA zone. This is in line with expectations because spleen has an important immune function and bigger dimensions are indicative of an infection response that could be caused by pesticides

exposure. In fact, splenic hyperplasia were already detected in mice after administration of some pesticides [192].

Significant differences were also found in relative weight liver for *Apodemus sylvaticus*, but this is not in line with expectations because animals from BA zone have bigger livers than CA zone. Liver is a filter organ, filtering xenobiotic agents and are involved on metabolism and detoxification of this compounds. So, it would be expected that animals from CA zone, exposed to pesticides, had a hyperplasic liver in order to provide as far as possible a satisfactory degradation of xenobiotics. This tendency for bigger livers in animals from BA zone only happened in the species *Apodemus sylvaticus* and it may be due to the presence of other xenobiotic agents.

Regarding hematological parameters, several studies have reported changes in those parameters as a consequence of pesticides exposure [192-194]. In the present study, significant results were only found for Hgb, MCH and MCHC parameters in *Mus spretus* species. These parameters are all related to hemoglobin and are higher in small mammals from CA zone that is not in line with expectations because pesticides seem to cause a decrease in hemoglobin content. It is important to refer that Beckman Coulter Act Diff Hematology Analyzer, the apparatus used to obtain these results, showed some inconsistencies in the provided values, so we must consider the hypothesis of these values are not the real ones.

In relation to genotype analysis, we were able to amplify *CYP1A1* in *Mus spretus* species but not in *Apodemus sylvaticus*. This is because the available primers were nor adequate to amplify this gene in *Apodemus sylvaticus* genome. Thus, to do an analysis of this kind, it is essential to start by drawing new primers that allowing the amplification of this genome region and then optimize the PCR technique as was done for *Mus spretus*. We only amplified the genome of ten *Mus spretus* from CA and BA zones and then only two samples were sequenced due to economic constraints. To obtain some reliable results it would be necessary to analyze much more individuals. However, comparing the two sequences amplified, one from CA zone and another from BA zone, it was found that they differ on only one base pair. So, this region of the genome seems to be very conservative between individuals and we cannot conclude that this difference is a SNP because we only compared two animals and this difference can be only genetic diversity between individuals of the same species.

Moreover, some samples were cut with two restriction enzymes, MseI and EcoRI, to research any possible polymorphism. These enzymes cut in the expected sites and there were no differences between animals from CA and BA zone. Only a small number of samples were tested due to economic constraint because enzymes are very expensive, so we cannot take conclusions.

It will be important to investigate specific polymorphisms of cytochrome P450 genes, like *CYP1A1*, involved in the xenobiotic´s metabolism because individuals with unfavorable metabolizing alleles can be more susceptible to genotoxic effects of xenobiotic agents, like pesticides, that can lead to an increased probability to developing cancer. Thus, the research of susceptibility biomarkers will be fundamental to the diagnosis and prevention of the disease.

In situ studies like the present that compares bioindicator species from a conventional agriculture zone *versus* a biological agriculture zone can be extremely important in assessing the toxicity of pesticides to humans. Even though the general population is not as exposed as animals because they only contact to pesticides through the consumption of agricultural products while animals live in the fields and are directly subjected to pesticide applications, the evaluation of the toxicological risk of pesticides in this way for humans is quite acceptable and more realistic than animal laboratory experiments. In fact, it is known that many pesticides are transformed in the environment through physical, chemical and biological processes which are intended to detoxify them but often the transformation process forms products that are more toxic than the parent [195]. Moreover, it is not feasible to predict the toxicity of pesticide mixtures on the basis of the results of the toxicity of single components because the effects of a mixture of pesticides are often different from the individual effects of a single components [196] and human exposure is rarely limited to a single compound. So, *in situ* studies are obviously closer to the real situation and must be considered in the pesticide approval process.

In conclusion, the present study clearly prove that conventional agriculture even with the use of pesticides legally approved in the European community, like Mancozeb, represent a higher genotoxic risk compared to biological agriculture. Despite all the controversy, biological agriculture has been adopted by an increasing number of farmers, like the farmer who collaborated in this study, and they believe that this practice has the potential for mass production of the amount of calories needed to feed humanity. We do not know if this is possible but it is urgent to find

and develop alternative and profitable agricultural practices, in order to minimize the use of pesticides, which represents a real global problem.

5. Final Remarks and Future Perspectives

- The present work contributes to alert about hazard effects resulting from pesticide exposure, particularly Mancozeb, a pesticide legally authorized for use in European Community;
- As revealed by the three cytogenetic endpoints used, Mancozeb is capable of inducing DNA damage in *Mus spretus* and *Apodemus sylvaticus* species;
- The use of several tests is essential to have a full picture of the genotoxic effects especially in wild species since the variability among animals can be higher than in laboratory animals;
- The use at least two sympatric species to detect the presence of genotoxic agents is essential because there may be a difference in sensitivity of the species selected as bioindicators in relation to the cytogenetic end points analyzed;
- Morphophisiological analysis was not able to detect pesticide toxicity. Only the increased spleens reveled clearly the consequences of pesticide exposure;
- AChE activity is not a good biomarker when the objective is to evaluate the long-term exposure to a mixture of pesticides, where some pesticides like Mancozeb can have components, which interfere with AChE activity;
- Mn quantification seems to be a good biomarker of exposure to Mancozeb and more research is needed to validate the metal quantifications as biomarkers of exposure to this type of pesticides;
- The present results provide reference values for the analyzed biomarkers allowing future comparisons with other small mammals' populations.
- *M. spretus* and *Apodemus sylvaticus* are suitable bioindicator species of pesticide pollution, demonstrating variation in morphological, biochemical and genotoxic effects of exposure;
- To determine *CYP1A1* polymorphisms, that could be genetic markers of susceptibility to genotoxic agents' action, it is necessary to continue the

preliminary genotype analysis described, analyzing more animals and exploring different primers and enzymes;

- Finally, despite Mancozeb toxicity, it will belong to the most widely used pesticide in the world due to their efficacy, and low relatively low cost and persistence in the environment. As a result, a large part of the population will continue to be exposed, and concerns for their known and potentially newly discovered adverse effects will remain alive.

6. References

[1] G.Y.H. McClure, R.M. Helm, K. Stine, A.W. Burks, S.M. Jones, J. Gandy, Evaluation of immune parameters in propanil-exposed farm families, Archives of Environmental Contamination and Toxicology, 41 (2001) 104-111.
[2] R. McKinlay, J.A. Plant, J.N.B. Bell, N. Voulvoulis, Calculating human exposure to endocrine disrupting pesticides via agricultural and non-agricultural exposure routes, Science of The Total Environment, 398 (2008) 1-12.
[3] A. Boobis, B. Ossendorp, U. Banasiak, P. Hamey, I. Sebestyen, A. Moretto, Cumulative risk assessment of pesticide residues in food, Toxicology Letters, 180 (2008) 137-150.
[4] FAO, International Code of Conduct on the Distribution and Use of Pesticides, Rome, 2005.
[5] M. Sanborn, D. Cole, K. Kerr, C. Vakil, L.H. Sanin, K. Bassil, Pesticides literature review. Chapter 1: Introduction, Ontario College of Family Physicians, Toronto, 2004. Available from: http://www.ocfp.on.ca/local/files/communications/current%20issues/pesticides/final%20paper%2023apr2004.pdf. Accessed January 2011.
[6] R. Carson, Silent Spring, 40th Anniversary edition, Houghton Mifflin, New York, 2002.
[7] A.J. Karabelas, K.V. Plakas, E.S. Solomou, V. Drossou, D.A. Sarigiannis, Impact of European legislation on marketed pesticides - A view from the standpoint of health impact assessment studies, Environment International, 35 (2009) 1096-1107.
[8] WHO, International Code of Conduct on the Distribution and Use of Pesticides: Guidelines for the Registration of Pesticides, Rome, 2010.
[9] F. Carvalho, Agriculture, pesticides, food security and food safety, Environmental Science & Policy, 9 (2006) 685-692.
[10] C. Bolognesi, G. Morasso, Genotoxicity of pesticides: potential risk for consumers, Trends in Food Science & Technology, 11 (2000) 182-187.
[11] Eurostat, Statistical Office of the European Communities, Luxemburg, 2011. Hyperlink to the graph: http://epp.eurostat.ec.europa.eu/tgm/graph.do?pcode=tag00084&language=en. Acessed July 2011.
[12] M. Fascendini, C. Colosio, S. Birindelli, M. Maroni, International centre for pesticides and health risk prevention, Pesticide Outlook, 13 (2002) 120-122.
[13] K. Vassilev, V. Kambourova, Pesticides as global environmental pollutants, in: L. Simeonov, E. Chirila (Eds.) Chemicals as intentional and accidental global environmental threats, Springer Netherlands, 2006, pp. 173-191.
[14] K. Sasaki, T. Ishizaka, T. Suzuki, M. Takeda, M. Uchiyama, Accumulation levels of organochlorine pesticides in human adipose tissue and blood, Bulletin of Environmental Contamination and Toxicology, 46 (1991) 662-669.
[15] K. Borgå, G.W. Gabrielsen, J.U. Skaare, Biomagnification of organochlorines along a Barents Sea food chain, Environmental Pollution, 113 (2001) 187-198.
[16] R. Bossi, P. Seiden, S.M. Andersen, C.S. Jacobsen, J.C. Streibig, Analysis of metsulfuron-methyl in soil by liquid chromatography/tandem mass spectrometry. Application to a field dissipation study, Journal of Agricultural and Food Chemistry, 47 (1999) 4462-4468.
[17] A.L. Aspelin, Pesticides industry sales and usage: 1994-95 market estimates, United States Environmental Protection Agency, Washington, 1997. Available from: http://www.epa.gov/opp00001/pestsales/95pestsales/market_estimates1995.pdf. Accessed July 2011.

[18] J. Cooper, H. Dobson, The benefits of pesticides to mankind and the environment, Crop Protection, 26 (2007) 1337-1348.
[19] Commission of the European Communities, Council Directive 91/414/EEC of 15 July 1991, Concerning the Placing of Plant Protection Products on the Market, Official Journal L 230, Brussels, 1991.
[20] EPA, Registering Pesticides, 2009. Available from: http://www.epa.gov/pesticides/regulating/index.htm. Accessed July 2011.
[21] H. Willer, L. Kilcher, The World of Organic Agriculture: statistics and emerging trends 2009. International Federation of Organic Agriculture Movements, Bonn. Research Institute of Organic Agriculture, Frick. International Trade Centre, Geneva, 2009. Available from: http://www.organic-world.net/fileadmin/documents/yearbook/2009/world-of-organic-agriculture-2009-small-2009-02-15.pdf#page=25. Accessed July 2011.
[22] L. Tamm, Organic agriculture: development and state of the art, Journal of Environmental Monitoring, 3 (2001) 92-96.
[23] T. Gomiero, D. Pimentel, M.G. Paoletti, Is there a need for a more sustainable agriculture?, Critical Reviews in Plant Sciences, 30 (2011) 6-23.
[24] WHO, The WHO Recommended Classification of Pesticides by Hazard, Geneva, 2010.
[25] EPA, Label Review Manual, Chapter 7: Precautionary Statements. Available from: http://www.epa.gov/oppfead1/labeling/lrm/chap-07.pdf. Accessed July 2011.
[26] O.N. Nesheim, F.M. Fishel, M. Mossler, Toxicity of Pesticides, Pesticide Information Office, Food Science and Human Nutrition Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, USA, 2008.
[27] W. Reifenrath, Enhanced Skin Absorption and Fly Toxicity of Permethrin in Emulsion Formulation, Bulletin of Environmental Contamination and Toxicology, 78 (2007) 299-303.
[28] W.A. Anwar, Biomarkers of human exposure to pesticides, Environmental Health Perspectives, 105 (1997) 801–806.
[29] IARC, Agents Reviewed by the IARC Monographs, Volumes 1-100, Available from: http://monographs.iarc.fr. Accessed August 2011.
[30] EPA, Evaluating Pesticides for Carcinogenic Potential, 2005.Available from: http://www.epa.gov/opp00001/health/cancerfs.htm. Accessed July 2011.
[31] C.A. Damalas, I.G. Eleftherohorinos, Pesticide Exposure, Safety Issues, and Risk Assessment Indicators, International Journal of Environmental Research and Public Health, 8 (2011) 1402-1419.
[32] C. Bolognesi, Genotoxicity of pesticides: a review of human biomonitoring studies, Mutation Research/Reviews in Mutation Research, 543 (2003) 251-272.
[33] S.M. Piperakis, K. Kontogianni, G. Karanastasi, Z. Iakovidou-Kritsi, A. Cebulska-Wasilewska, M.M. Piperakis, Investigation of the genotoxic effect of pesticides on greenhouse workers' lymphocytes, Environmental and Molecular Mutagenesis, 50 (2009) 121-126.
[34] S. Bull, Evidence for genotoxicity of pesticides in pesticide applicators: a review, Mutagenesis, 21 (2006) 93-103.
[35] M. Nasterlack, Do pesticides cause childhood cancer?, International Archives of Occupational and Environmental Health, 79 (2006) 536-544.
[36] R. Stiller-Winkler, W. Hadnagy, G. Leng, E. Straube, H. Idel, Immunological parameters in humans exposed to pesticides in the agricultural environment, Toxicology Letters, 107 (1999) 219-224.

[37] M.V. Nunes, E.H. Tajara, Efeitos tardios dos praguicidas organoclorados no homem, Revista de Saúde Pública, 32 (1998) 372-382.
[38] K.L. Bassil, C. Vakil, M. Sanborn, D.C. Cole, J.S. Kaur, K.J. Kerr, Cancer health effects of pesticides, Canadian Family Physician, 53 (2007) 1704-1711.
[39] R. Betarbet, T.B. Sherer, G. MacKenzie, M. Garcia-Osuna, A.V. Panov, J.T. Greenamyre, Chronic systemic pesticide exposure reproduces features of Parkinson's disease, Nature Neuroscience, 3 (2000) 1301-1306.
[40] M. Bjørling-Poulsen, H. Andersen, P. Grandjean, Potential developmental neurotoxicity of pesticides used in Europe, Environmental Health, 7 (2008) 50.
[41] M.S. García-Ayllón, I. Riba-Llena, C. Serra-Basante, J. Alom, R. Boopathy, J. Sáez-Valero, Altered levels of acetylcholinesterase in alzheimer plasma, PLoS ONE, 5 (2010) e8701.
[42] S. Mena, A. Ortega, J.M. Estrela, Oxidative stress in environmental-induced carcinogenesis, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 674 (2009) 36-44.
[43] D. Bagchi, M. Bagchi, E.A. Hassoun, S.J. Stohs, In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides, Toxicology, 104 (1995) 129-140.
[44] M. Abdollahi, A. Ranjbar, S. Shadnia, S. Nikfar, A. Rezaie, Pesticides and oxidative stress: a review, Medical Science Monitore, 10 (2004) 141-147.
[45] B.D. Banerjee, V. Seth, R.S. Ahmed, Pesticide-induced oxidative stress: perspectives and trends, Reviews on Environmental Health, 16 (2001) 1-40.
[46] J.D. Robertson, S. Orrenius, Molecular mechanisms of apoptosis induced by cytotoxic chemicals, Critical Reviews in Toxicology, 30 (2000) 609-627.
[47] L. Galluzzi, M.C. Maiuri, I. Vitale, H. Zischka, M. Castedo, L. Zitvogel, G. Kroemer, Cell death modalities: classification and pathophysiological implications, Cell Death Differ, 14 (2007) 1237-1243.
[48] R. Franco, R. Sánchez-Olea, E.M. Reyes-Reyes, M.I. Panayiotidis, Environmental toxicity, oxidative stress and apoptosis: Ménage à Trois, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 674 (2009) 3-22.
[49] J. Blasiak, P. Jaloszynski, A. Trzeciak, K. Szyfter, In vitro studies on the genotoxicity of the organophosphorus insecticide malathion and its two analogues, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 445 (1999) 275-283.
[50] P. Houeto, G. Bindoula, J.R. Hoffman, Ethylenebisdithiocarbamates and ethylenethiourea: possible human health hazards, Environmental Health Perspectives, 103 (1995) 568-573.
[51] EPA, Reregistration Eligibility Decision for Mancozeb, 2005.
[52] M. Maroni, C. Colosio, A. Ferioli, A. Fait, Biological Monitoring of Pesticide Exposure: a review, Toxicology, 143 (2000) 1-118.
[53] WHO, Dithiocarbamate pesticides, ethylenethiourea, and propylenethiourea: a general introduction, Environmental Health Criteria, Geneva, 1988.
[54] K. Atreya, B.K. Sitaula, Mancozeb: growing risk for agricultural communities?, Himalayan Journal of Sciences, 6 (2010) 9-10.
[55] W.H. Newsome, G.W. Laver, Effect of boiling on the formation of ethylenethiourea in zineb-treated foods, Bulletin of Environmental Contamination and Toxicology, 10 (1973) 151-154.

[56] R.S. Chhabra, S. Eustis, J.K. Haseman, P.J. Kurtz, B.D. Carlton, Comparative Carcinogenicity of Ethylene Thiourea with or without Perinatal Exposure in Rats and Mice, Toxicological Sciences, 18 (1992) 405-417.
[57] C. Nebbia, J. Fink-Gremmels, Acute effects of low doses of Zineb and Ethylenethiourea on thyroid function in the male rat, Bulletin of Environmental Contamination and Toxicology, 56 (1996) 847-852.
[58] K. Steenland, L. Cedillo, J. Tucker, C. Hines, K. Sorensen, J. Deddens, V. Cruz, Thyroid hormones and cytogenetic outcomes in backpack sprayers using ethylenebis(dithiocarbamate) (EBDC) fungicides in Mexico, Environmental Health Perspectives, 105 (1997) 1126-1130.
[59] R. Kackar, M.K. Srivastava, R.B. Raizada, Induction of gonadal toxicity to male rats after chronic exposure to mancozeb, Industrial Health, 35 (1997) 104-111.
[60] E. Corsini, S. Birindelli, S. Fustinoni, G. De Paschale, T. Mammone, S. Visentin, C.L. Galli, M. Marinovich, C. Colosio, Immunomodulatory effects of the fungicide Mancozeb in agricultural workers, Toxicology and Applied Pharmacology, 208 (2005) 178-185.
[61] P.N. Baligar, B.B. Kaliwal, Induction of gonadal toxicity to female rats after chronic exposure to mancozeb, Industrial Health, 39 (2001) 235-243.
[62] G. Rossi, R. Buccione, M. Baldassarre, G. Macchiarelli, M. Palmerini, S. Cecconi, Mancozeb exposure in vivo impairs mouse oocyte fertilizability, Reproductive Toxicology, 21 (2006) 216-219.
[63] G. Rossi, M. Palmerini, G. Macchiarelli, R. Buccione, S. Cecconi, Mancozeb adversely affects meiotic spindle organization and fertilization in mouse oocytes, Reproductive Toxicology, 22 (2006) 51-55.
[64] B.B. Bindali, B.B. Kaliwal, Anti-implantation Effect of a Carbamate Fungicide Mancozeb in Albino Mice Industrial Health, 40 (2002) 191-197.
[65] L. Miranda-Contreras, R. Dávila-Ovalles, P. Benítez-Díaz, Z. Peña-Contreras, E. Palacios-Prü, Effects of prenatal paraquat and mancozeb exposure on amino acid synaptic transmission in developing mouse cerebellar cortex, Developmental Brain Research, 160 (2005) 19-27.
[66] F. Belpoggi, M. Soffritti, M. Guarino, L. Lambertini, D. Cevolani, C. Maltoni, Results of Long-Term Experimental Studies on the Carcinogenicity of Ethylene-bis-Dithiocarbamate (Mancozeb) in Rats, Annals of the New York Academy of Sciences, 982 (2002) 123-136.
[67] G. Calviello, E. Piccioni, A. Boninsegna, B. Tedesco, N. Maggiano, S. Serini, F.I. Wolf, P. Palozza, DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: Involvement of the oxidative mechanism, Toxicology and Applied Pharmacology, 211 (2006) 87-96.
[68] B.P. Varnagy, E. Molnar, I. Takacs, A. Karpati, Interaction of dithane M-45(mancozeb) and lead acetate during a teratogenicity test in rats, Acta Veterinaria Hungara, 48 (2000) 113-124.
[69] M. González, S. Soloneski, M.A. Reigosa, M.L. Larramendy, Effect of dithiocarbamate pesticide zineb and its commercial formulation, azzurro: IV. DNA damage and repair kinetics assessed by single cell gel electrophoresis (SCGE) assay on Chinese hamster ovary (CHO) cells, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 534 (2003) 145-154.
[70] V.A. Fitsanakis, V. Amarnath, J.T. Moore, K.S. Montine, J. Zhang, T.J. Montine, Catalysis of catechol oxidation by metal-dithiocarbamate complexes in pesticides, Free Radical Biology and Medicine, 33 (2002) 1714-1723.

[71] WHO/IPCS, Environmental health criteria 155. Biomarkers and risk assessment: Concepts and principles, Geneva, 1993.
[72] M. Dusinska, A.R. Collins, The comet assay in human biomonitoring: gene–environment interactions, Mutagenesis, 23 (2008) 191-205.
[73] H. Vainio, Molecular approaches in toxicology: change in perspective, Journal of Occupational and Environmental Medicine, 37 (1995) 14-18.
[74] Committee on Advances in Assessing Human Exposure to Airborne Pollutants, Human Exposure Assessment For Airborne Pollutants: Advances and Opportunities, National Academy of Sciences, Washington (1991), pp.115-142. Available from: http://www.nap.edu/openbook.php?record_id=1544&page=117. Accessed July 2011.
[75] D.B. Barr, L.L. Needham, Analytical methods for biological monitoring of exposure to pesticides: a review, Journal of Chromatography B, 778 (2002) 5-29.
[76] C. Aprea, C. Colosio, T. Mammone, C. Minoia, M. Maroni, Biological monitoring of pesticide exposure: a review of analytical methods, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 769 (2002) 191-219.
[77] S. Pavanello, E. Clonfero, Biological indicators of genotoxic risk and metabolic polymorphisms, Mutation Research/Reviews in Mutation Research, 463 (2000) 285-308.
[78] L. Hagmar, S. Bonassi, U. Strömberg, Z. Mikoczy, C. Lando, I.-L. Hansteen, A.H. Montagud, L. Knudsen, H. Norppa, C. Reuterwall, H. Tinnerberg, A. Brøgger, A. Forni, B. Högstedt, B. Lambert, F. Mitelman, I. Nordenson, S. Salomaa, S. Skerfving, Cancer predictive value of cytogenetic markers used in occupational health surveillance programs: a report from an ongoing study by the European Study Group on Cytogenetic Biomarkers and Health, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 405 (1998) 171-178.
[79] M. Sanborn, D. Cole, K. Kerr, C. Vakil, L.H. Sanin, K. Bassil, Pesticides literature review. Chapter 6: Genotoxicity, Ontario College of Family Physicians, Toronto, 2004. Available from:
http://www.ocfp.on.ca/local/files/communications/current%20issues/pesticides/final%20paper%2023apr2004.pdf. Accessed January 2011.
[80] L. Forchhammer, E.V. Bräuner, J.K. Folkmann, P.H. Danielsen, C. Nielsen, A. Jensen, S. Loft, G. Friis, P. Møller, Variation in assessment of oxidatively damaged DNA in mononuclear blood cells by the comet assay with visual scoring, Mutagenesis, 23 (2008) 223-231.
[81] F. Faust, F. Kassie, S. Knasmüller, R.H. Boedecker, M. Mann, V. Mersch-Sundermann, The use of the alkaline comet assay with lymphocytes in human biomonitoring studies, Mutation Research/Reviews in Mutation Research, 566 (2004) 209-229.
[82] W. Liao, M.A. McNutt, W.-G. Zhu, The comet assay: A sensitive method for detecting DNA damage in individual cells, Methods, 48 (2009) 46-53.
[83] A. Dhawan, M. Bajpayee, D. Parmar, Comet assay: a reliable tool for the assessment of DNA damage in different models, Cell Biology and Toxicology, 25 (2009) 5-32.
[84] O. Ostling, K.J. Johanson, Microelectrophoretic study of radiationinduced DNA damages in individual mammalian cells, Biochemical and Biophysical Research Communications 123 (1984) 291-298.
[85] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing, Environmental and Molecular Mutagenesis, 35 (2000) 206-221.

[86] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Experimental Cell Research, 175 (1988) 184-191.
[87] A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud, R.R. Tice, Recommendations for conducting the in vivo alkaline Comet assay, Mutagenesis, 18 (2003) 45-51.
[88] G. Speit, A. Hartman, The comet assay (single-cell gel test): a sensitive genotoxicity test for the detection of DNA damage and repair, DNA Repair Protocols, 113 (1999) 203-212.
[89] A. Collins, M. Dušinská, M. Franklin, M. Somorovská, H. Petrovská, S. Duthie, L. Fillion, M. Panayiotidis, K. Rašlová, N. Vaughan, Comet assay in human biomonitoring studies: Reliability, validation, and applications, Environmental and Molecular Mutagenesis, 30 (1997) 139-146.
[90] A. Collins, The comet assay for DNA damage and repair, Molecular Biotechnology, 26 (2004) 249-261.
[91] P. Møller, Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA, Mutation Research/Reviews in Mutation Research, 612 (2006) 84-104.
[92] A.R. Collins, V.L. Dobson, M. Duinská, G. Kennedy, R. ttina, The comet assay: what can it really tell us?, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 375 (1997) 183-193.
[93] T. Kumaravel, B. Vilhar, S. Faux, A. Jha, Comet Assay measurements: a perspective, Cell Biology and Toxicology, 25 (2009) 53-64.
[94] S.W. Maluf, B. Erdtmann, Genomic instability in Down syndrome and Fanconi anemia assessed by micronucleus analysis and single-cell gel electrophoresis, Cancer Genetics and Cytogenetics, 124 (2001) 71-75.
[95] S. Brendler-Schwaab, A. Hartmann, S. Pfuhler, G. Speit, The in vivo comet assay: use and status in genotoxicity testing, Mutagenesis, 20 (2005) 245-254.
[96] S. Sato, I. Tomita, Short-term screening method for the prediction of carcinogenicity of chemical substances: current status and problems of an in vivo rodent micronucleus assay Journal of Health Science, 47 (2001) 1-8.
[97] G. Krishna, M. Hayashi, In vivo rodent micronucleus assay: protocol, conduct and data interpretation, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 455 (2000) 155-166.
[98] G. Iarmarcovai, S. Bonassi, A. Botta, R.A. Baan, T. Orsière, Genetic polymorphisms and micronucleus formation: A review of the literature, Mutation Research/Reviews in Mutation Research, 658 (2008) 215-233.
[99] W. Schmid, The micronucleus test, Mutation Research, 31 (1975) 9-15.
[100] M. Hayashi, T. Morita, Y. Kodama, T. Sofuni, M. Ishidate Jr, The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides, Mutation Research Letters, 245 (1990) 245-249.
[101] P. Becker, H. Scherthan, H. Zankl, Use of a centromere-specific DNA probe (p82H) in nonisotopic in situ hybridization for classification of micronuclei, Genes Chromosomes and Cancer, 2 (1990) 59-62.
[102] B.M. Miller, H.F. Zitzelsberger, H.-U.G. Weier, I.-D. Adler, Classification of micronuclei in murine erythrocytes: immunofluorescent staining using CREST antibodies compared to in situ hybridization with biotinylated gamma satellite DNA, Mutagenesis, 6 (1991) 297-302.
[103] R. Bretveld, M. Brouwers, I. Ebisch, N. Roeleveld, Influence of pesticides on male fertility, Scandinavian Journal of Work, Environment and Health, 33 (2007) 13-28.

[104] H.R. Contreras, V. Paredes, B. Urquieta, L. Del Valle, E. Bustos-Obregón, Testosterone production and spermatogenic damage induced by organophosphorate pesticides, Biocell, 30 (2006) 423-429.
[105] L.C. Sánchez-Peña, B.E. Reyes, L. López-Carrillo, R. Recio, J. Morán-Martínez, M.E. Cebrián, B. Quintanilla-Vega, Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers, Toxicology and Applied Pharmacology, 196 (2004) 108-113.
[106] S. Yucra, M. Gasco, J. Rubio, G.F. Gonzales, Semen quality in Peruvian pesticide applicators: association between urinary organophosphate metabolites and semen parameters, Environmental Health, 7 (2008) 59.
[107] A. Okamura, M. Kamijima, K. Ohtani, O. Yamanoshita, D. Nakamura, Y. Ito, M. Miyata, J. Ueyama, T. Suzuki, R. Imai, K. Takagi, T. Nakajima, Broken sperm, cytoplasmic droplets and reduced sperm motility are principal markers of decreased sperm quality due to organophosphorus pesticides in rats, Journal of Occupational Health, 51 (2009) 478-487.
[108] M.J. Perry, Effects of environmental and occupational pesticide exposure on human sperm: a systematic review, Human Reproduction Update, 14 (2008) 233-242.
[109] D. Whorton, R. Krauss, S. Marshall, T. Milby, Infertility in male pesticide workers, The Lancet, 310 (1977) 1259-1261.
[110] S. Perreault, A. Cancel, Significance of incorporating measures of sperm production and function into rat toxicology studies, Reproduction, 121 (2001) 207-216.
[111] S. Padmalatha Rai, K.K. Vijayalaxmi, Tamoxifen citrate induced sperm shape abnormalities in the in vivo mouse, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 492 (2001) 1-6.
[112] A.C.C. Rios, D.M.F. Salvadori, S.V. Oliveira, L.R. Ribeiro, The action of the herbicide paraquat on somatic and germ cells of mice, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 328 (1995) 113-118.
[113] A.J. Wyrobek, W.R. Bruce, Chemical induction of sperm abnormalities in mice, Proceedings of the National Academy of Sciences, 72 (1975) 4425-4429.
[114] J.C. Topham, Chemically-induced transmissible abnormalities in sperm-head shape, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 70 (1980) 109-114.
[115] J.C. Topham, The detection of carcinogen-induced sperm head abnormalities in mice, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 69 (1980) 149-155.
[116] W.R. Bruce, R. Furrer, A.J. Wyrobek, Abnormalities in the shape of murine sperm after acute testicular X-irradiation, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 23 (1974) 381-386.
[117] M.J. Hegde, T.V. Sujatha, In-vivo genotoxicity of the alkaloid drug pilocarpine nitrate in bone marrow cells and male germ cells of mice, Mutation Research/Genetic Toxicology, 344 (1995) 103-108.
[118] A. Fairbrother, J. Bennett, The usefulness of cholinesterase measurements, Journal of Wildlife Diseases, 24 (1988) 587-593.
[119] T.R. Fukuto, Mechanism of action of organophosphatrus and carbamate insecticides, Environmental Health Perspectives, 14 (1990) 245-254.
[120] D. Milatovic, R.C. Gupta, M. Aschner, Anticholinesterase toxicity and oxidative stress, Scientific World Journal, 6 (2006) 295-310.
[121] FAO, Pesticides in food report 2007, FAO Plant Production and Protection Paper 191, Rome, 2007.

[122] S. Darvesh, D.A. Hopkins, C. Geula, Neurobiology of butyrylcholinesterase, Nature Reviews Neuroscience, 4 (2003) 131-138.
[123] M. Lotti, Cholinesterase inhibition: complexities in interpretation, Clinical Chemistry, 41 (1995) 1814-1818.
[124] V.K. Singh, Jyoti, M.M.K. Reddy, C. Kesavachandran, S.K. Rastogi, M.K.J. Siddiqui, Biomonitoring of organochlorines, glutathione, lipid peroxidation and cholinesterase activity among pesticide sprayers in mango orchards, Clinica Chimica Acta, 377 (2007) 268-272.
[125] S. Singh, V. Kumar, S. Thakur, B.D. Banerjee, S. Chandna, R.S. Rautela, S.S. Grover, D.S. Rawat, S.T. Pasha, S. Jain, R.L. Ichhpujani, A. Rai, DNA damage and cholinesterase activity in occupational workers exposed to pesticides, Environmental toxicology and Pharmacology, 31 (2011) 278-285.
[126] A.P. Remor, C.C. Totti, D.A. Moreira, G.P. Dutra, V.D. Heuser, J.M. Boeira, Occupational exposure of farm workers to pesticides: Biochemical parameters and evaluation of genotoxicity, Environment International, 35 (2009) 273-278.
[127] Y. Miao, N. He, J.J. Zhu, History and New Developments of Assays for Cholinesterase Activity and Inhibition, Chemical Reviews, 110 (2010) 5216-5234.
[128] ASTDR, Toxicological profile for manganese, US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, Atlanta Georgia, 2000.
[129] G.B. Gerber, A. Léonard, P. Hantson, Carcinogenicity, mutagenicity and teratogenicity of manganese compounds, Critical Reviews in Oncology/Hematology, 42 (2002) 25-34.
[130] D.G. Barceloux, Manganese, Journal of Toxicology/Clinical Toxicology, 37 (1999) 293-307.
[131] J. Couper, On the effects of black oxide of manganese when inhaled into the lungs, British annual of Medicine and Pharmacology, 1(1837) 41-42 in A.B. Santamaria, Manganese exposure, essentiality & toxicity, Indian Journal of Medical Research, 128 (2008) 484-500.
[132] C. Santos-Burgoa, C. Rios, L.A. Mercado, R. Arechiga-Serrano, F. Cano-Valle, R.A. Eden-Wynter, J.L. Texcalac-Sangrador, J.P. Villa-Barragan, Y. Rodriguez-Agudelo, S. Montes, Exposure to manganese: health effects on the general population, a pilot study in Central Mexico, Environmental Research, 85 (2001) 90-104.
[133] WHO, Manganese and its compounds: environmental aspects, Geneva, 2004.
[134] R. Migheli, C. Godani, L. Sciola, M.R. Delogu, P.A. Serra, D. Zangani, G. De Natale, E. Miele, M.S. Desole, Enhancing Effect of Manganese on L-DOPA-Induced Apoptosis in PC12 Cells, Journal of Neurochemistry, 73 (1999) 1155-1163.
[135] A.P. Marreilha dos Santos, D. Santos, C. Au, D. Milatovic, M. Aschner, M.C.C. Batoréu, Antioxidants prevent the cytotoxicity of manganese in RBE4 cells, Brain Research, 1236 (2008) 200-205.
[136] H. Martinez, E. Bonilla, Water intake and brain choline-acetyltransferase and acetylcholinesterase activites in manganese treated rats, Neurobehavioral Toxicology and Teratology 3(1981) 277-280.
[137] C. Liapi, A. Zarros, P. Galanopoulou, S. Theocharis, N. Skandali, H. Al-Humadi, F. Anifantaki, E. Gkrouzman, Z. Mellios, S. Tsakiris, Effects of short-term exposure to manganese on the adult rat brain antioxidant status and the activities of acetylcholinesterase, (Na,K)-ATPase and Mg-ATPase: modulation by L-cysteine, Basic and Clinical Pharmacology and Toxicology, 103 (2008) 171-175.
[138] Y. Finkelstein, D. Milatovic, M. Aschner, Modulation of cholinergic systems by manganese, NeuroToxicology, 28 (2007) 1003-1014.

[139] J.K.C. Lai, T.K.C. Leung, L. Lim, Brain regional distribution of glutamic acid decarboxylase, choline acetyltransferase, and acetylcholinesterase in the rat: effects of chronic manganese chloride administration after two years, Journal of Neurochemistry, 36 (1981) 1443-1448.
[140] J.K.C. Lai, T.K.C. Leung, L. Lim, Differences in the neurotoxic effects of manganese during development and aging: some observations on brain regional neurotransmitter and non-neurotransmitter metabolism in a developmental rat model of chronic manganese encephalopathy, NeuroToxicology, 5 (1984) 37-47.
[141] J.K.C. Lai, A.W.K. Chan, T.K.C. Leung, M.J. Minski, L. Lira, Neurochemical Changes in Rats Chronically Treated with a High Concentration of Manganese Chloride, Neurochemical Research, 17 (1992) 841-847.
[142] L. Alessio, P. Apostoli, A. Ferioli, S. Lombardi, Interference of Manganese on neuroendocrinal system in exposed workers, Biological Trace Element Research, 21 (1989) 249-253.
[143] R.M. Bowler, H.A. Roels, S. Nakagawa, M. Drezgic, E. Diamond, R. Park, W. Koller, R.P. Bowler, D. Mergler, M. Bouchard, D. Smith, R. Gwiazda, R.L. Doty, Dose–effect relationships between manganese exposure and neurological, neuropsychological and pulmonary function in confined space bridge welders, Occupational and Environmental Medicine, 64 (2007) 167-177.
[144] I. Figà-Talamanca, M.E. Traina, E. Urbani, Occupational exposures to metals, solvents and pesticides: recent evidence on male reproductive effects and biological markers, Occupational Medicine, 51 (2001) 174-188.
[145] F. Baruthio, O. Guillard, J. Arnaud, F. Pierre, R. Zawislak, Determination of manganese in biological materials by electrothermal atomic absorption spectrometry: a review, Clin Chem, 34 (1988) 227-234.
[146] J. Versieck, L. Vanballenberghe, A. De Kesel, Accuracy of biological trace-element determination, Biological Trace Element Research, 12 (1987) 45-54.
[147] L. Kruglyak, D.A. Nickerson, Variation is the spice of life, Nature Genetics, 3 (2001) 234-236.
[148] J.C. Barrett, H. Vainio, D. Peakall, D. Bernard, B.D. Goldstein, 12th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals: Susceptibility to Environmental Hazards, Environmental Health Perspectives, 105 (1997) 694-737.
[149] L. Rosival, T. Trnovec, Gene-environment interactions (A challenge for the future), Central European Journal of Occupational and environmental Medicine, 5 (1999) 3-16.
[150] T. Omura, Forty Years of Cytochrome P450, Biochemical and Biophysical Research Communications, 266 (1999) 690-698.
[151] Y. Gueguen, K. Mouzat, L. Ferrari, E. Tissandie, J.M.A. Lobaccaro, A.M. Batt, F. Paquet, P. Voisin, J. Aigueperse, P. Gourmelon, M. Souidi, Cytochromes P450: xenobiotic metabolism, regulation and clinical importance, Annales De Biologie Clinique, 64 (2006) 535-548.
[152] D.W. Nebert, D.W. Russell, Clinical importance of the cytochromes P450, The Lancet, 360 (2002) 1155-1162.
[153] D. Koop, Oxidative and reductive metabolism by cytochrome P450 2E1, The FASEB Journal, 6 (1992) 724-730.
[154] S.G. Kim, R.F. Novak, Role of P450IIE1 in the metabolism of 3-Hydroxypyridine, a constituent of tobacco smoke: redox cycling and DNA strand scission by the metabolite 2,5-Dihydroxypyridine, Cancer Research, 50 (1990) 5333-5339.

[155] K. Kawajiri, H. Eguchi, K. Nakachi, T. Sekiya, M. Yamamoto, Association of CYP1A1 germ line polymorphisms with mutations of the p53 gene in lung cancer, Cancer Research, 56 (1996) 72-76.
[156] L. Sivaraman, M.P. Leatham, J. Yee, L.R. Wilkens, A.F. Lau, L.L. Marchand, CYP1A1 genetic polymorphisms and in situ colorectal cancer, Cancer Research, 54 (1994) 3692-3695.
[157] E. Taioli, J. Trachman, X. Chen, P. Toniolo, S.J. Garte, A CYP1A1 restriction fragment length polymorphism Is associated with breast cancer in African-American women, Cancer Research, 55 (1995) 3757-3758.
[158] A. Musio, I. Sbrana, Aphidicolin-sensitive specific common fragile sites: A biomarker of exposure to pesticides, Environmental and Molecular Mutagenesis, 29 (1997) 250-255.
[159] P.B. Landres, J. Verner, J.W. Thomas, Ecological uses of vertebrate indicator species: a critique, Conservation Biology, 2 (1988) 316-328.
[160] C. Tanzarella, F. Degrassi, M. Cristaldi, S. Moreno, A. Lascialfari, G. Chiuchiarelli, L.A. Ieradi, Genotoxic damage in free-living Algerian mouse (Mus spretus) after the Coto Doñana ecological disaster, Environmental Pollution, 115 (2001) 43-48.
[161] S.S. Talmage, B.T. Walton, Small mammals as monitors of environmental contaminants, Review of Environmental Contamination and Toxicology, 119 (1991) 47-108.
[162] A.C. Nunes, M. da Luz Mathias, A.M. Crespo, Morphological and haematological parameters in the Algerian mouse (Mus spretus) inhabiting an area contaminated with heavy metals, Environmental Pollution, 113 (2001) 87-93.
[163] M. Velickovic, Measures of the developmental stability, body size and body condition in the black-striped mouse (Apodemus agrarius) as indicators of a disturbed environment in northern Serbia, Belgian Journal of Zoology, 137 (2007) 147-156.
[164] K. Tersago, W. De Coen, J. Scheirs, K. Vermeulen, R. Blust, D. Van Bockstaele, R. Verhagen, Immunotoxicology in wood mice along a heavy metal pollution gradient, Environmental Pollution, 132 (2004) 385-394.
[165] K. McBee, J.W. Bickham, Petrol-chemical related DNA damage in wild rodents detected by flow cytometry, Bulletin of Environmental Contamination and Toxicology 40 (1988) 343-349.
[166] R. Metcheva, S. Teodorova, M. Topashka-Ancheva, A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria I: monitoring points and bioaccumulation features, Ecotoxicology and Environmental Safety, 54 (2003) 176-187.
[167] S.R. Sheffield, K. Sawiska-Kapusta, J.B.Cohen, B.A. Rattner, Rodentia and Lagomorpha, in: R.F. Shore, B.A. Rattner, (Eds.), Ecotoxicology of Wild mammals, Ecological and Environmental Toxicology Series, Chichester, 2001, pp. 215-314.
[168] W.C. Ma, S.S. Talmage, Insectivora, in: R.F. Shore, B.A. Rattner, (Eds.), Ecotoxicology of Wild mammals, Ecological and Environmental Toxicology Series, John Wiley and Sons Limited, Chichester, 2001, pp. 123-158.
[169] L.A. Ieradi, S. Moreno, J.P. Bolívar, A. Cappai, A. Di Benedetto, M. Cristaldi, Free-living rodents as bioindicators of genetic risk in natural protected areas, Environmental Pollution, 102 (1998) 265-268.
[170] L.A. Ieradi, M. Cristaldi, D. Mascanzoni, E. Cardarelli, R. Grossi, L. Campanella, Genetic damage in urban mice exposed to traffic pollution, Environmental Pollution, 92 (1996) 323-328.
[171] A. Gorriz, S. Llacuna, M. Riera, J. Nadal, Effects of air pollution on hematological and plasma parameters in *Apodemus sylvaticus* and *Mus musculus*, Archives of Environmental Contamination and Toxicology, 31 (1996) 153-158.

[172] M.L. Mathias, Guia do mamíferos terrestres de Portugal Continental, Açores e Madeira, Instituto da Conservação da Natureza, Lisboa, 1999.
[173] L.J. Palomo, J. Gisbert, 2002. Atlas de los mamíferos terrestres de España. Dirección General de Conservación de la Natureza – SECEM- SECEMU, Madrid.
[174] Image available from http://www.bobperryonline.co.uk/. Accessed July 2011.
[175] Image available from: http://www.biodiversitat.cat/Fitxes_mamifers/Fitxa_mus_spretus .htm. Accessed July 2011.
[176] National Research Council (NRC), 1991. Animals as sentinels of environmental health hazards, 1st Edition, National Academy Press, Washington, DC, USA.
[177] Image available from: http://www.pbase.com/joseelias/image/112065334. Accessed August 2011.
[178] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimental purposes, Official Journal of the European Union. Available from: http://ec.europa.eu/food/animal/welfare/references_en.htm. Accessed July 2010.
[179] U.R. Acharya, R.M. Rathore, M. Mishra, Role of vitamin C on lead acetate induced spermatogenesis in swiss mice, Environmental Toxicology and Pharmacology, 13 (2003) 9-14.
[180] G.L. Ellman, K.D. Courtney, V. Andreas, Jr., R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochemical Pharmacology, 7 (1961) 88-90.
[181] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72 (1976) 248-254.
[182] A. Dey, D.W. Nebert, Markedly Increased Constitutive CYP1A1 mRNA Levels in the Fertilized Ovum of the Mouse, Biochemical and Biophysical Research Communications, 251 (1998) 657-661.
[183] C.A. Damalas, Understanding benefits and risks of pesticide use, Scientific Research and Essay, 4 (2009) 945-949.
[184] A. Medjdoub, S.A. Merzouk, H. Merzouk, F.Z. Chiali, M. Narce, Effects of Mancozeb and Metribuzin on in vitro proliferative responses and oxidative stress of human and rat spleen lymphocytes stimulated by mitogens, Pesticide Biochemistry and Physiology, 101 (2011) 27-33.
[185] P.K. Khan, S.P. Sinha, Ameliorating effect of vitamin C on murine sperm toxicity induced by three pesticides (endosulfan, phosphamidon and mancozeb), Mutagenesis, 11 (1996) 33-36.
[186] E. Canossa, G. Angiuli, G. Garasto, A. Buzzoni, E. De Rosa, Dosage indicators in farm workers exposed to mancozeb, La Medicina del lavoro, 84 (1993) 42-50.
[187] A.M.S. Bueno, C.A.d.B. Pereira, M.N. Rabello-Gay, Environmental genotoxicity evaluation using cytogenetic end points in wild rodents, Environ Health Perspect, 108 (2000).
[188] U.A. Meyer, U.M. Zanger, Molecular mechanisms of genetic polymorphisms of drug metabolism, Annual Review of Pharmacology and Toxicology, 37 (1997) 269-296.
[189] V.D. Heuser, V.M. de Andrade, A. Peres, L.M. Gomes de Macedo Braga, J.A. Bogo Chies, Influence of age and sex on the spontaneous DNA damage detected by Micronucleus test and Comet assay in mice peripheral blood cells, Cell Biology International, 32 (2008) 1223-1229.
[**190]** F. Van Goethem, D. Lison, M. Kirsch-Volders, Comparative evaluation of the in vitro micronucleus test and the alkaline single cell gel electrophoresis assay for the detection of

DNA damaging agents: genotoxic effects of cobalt powder, tungsten carbide and cobalt-tungsten carbide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 392 (1997) 31-43.

[191] I. Kim, C. Hyun, Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms, Ecotoxicology and Environmental Safety, 64 (2006) 288-297.

[192] M. Gupta, G. Bagchi, S. Bandyopadhyay, D. Sasmal, T. Chatterjee, S.N. Dey, Hematological changes produced in mice by Nuvacron or Furadan, Toxicology, 25 (1982) 255-260.

[193] R. Badraoui, N.B. Abdelmoula, T. Rebai, Erythrocytes oxidative damage and hematological effects of 2,4,4′,5-tetrachlorodiphenyl sulfone in rats, Experimental and Toxicologic Pathology, 63 (2011) 479-482.

[194] I. Celik, H. Suzek, The hematological effects of methyl parathion in rats, Journal of Hazardous Materials, 153 (2008) 1117-1121.

[195] C.J. Sinclair, A.B.A. Boxall, Assessing the Ecotoxicity of Pesticide Transformation Products, Environmental Science & Technology, 37 (2003) 4617-4625.

[196] M. Marinovich, F. Ghilardi, C.L. Galli, Effect of pesticide mixtures on in vitro nervous cells: Comparison with single pesticides, Toxicology, 108 (1996) 201-206.

Chapter II

In situ Assessment of Mancozeb Genotoxicity using small mammals as bioindicators: Comparison between Conventional and Biological Agriculture

Vânia Gaio[a]*, Ana Sofia Boa-Alma[a], Deodália Dias[a,c] , Ana Paula Marreilha dos Santos[d] & Maria da Graça Ramalhinho [b,c]

[a] Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Edifício C2, 3º piso, Campo Grande, 1749-016 Lisboa, Portugal;

[b] Museu Nacional de História Natural (Museu Bocage), Rua da Escola Politécnica, 58, 1268-102 Lisboa, Portugal;

[c] Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal;

[d] i-Med.UL, Departamento de Toxicologia e Ciências dos Alimentos, Faculdade de Farmácia da Universidade de Lisboa, Avenida Professor Gama Pinto, 1649-003 Lisboa, Portugal.

*Corresponding author: Tel.: + 351 217500000; Fax: +351 217500028
E-mail address: vaniagaio@hotmail.com (Vânia Gaio).

Abstract

Pesticides are widely used for pest control in conventional agriculture, despite their negative impacts. Biological agriculture can represent a valuable option, avoiding the use of pesticides, but it does not appear to have the potential to respond to the mass production of food to feed humanity.

Mancozeb is a widely used fungicide mainly because of its low acute toxicity in mammals and scarce persistence in the environment. However, it has been considered a multipotent carcinogen, mutagenic and possibly teratogenic in a long-term exposure context.

Small mammals can be used as bioindicators of environmental toxicity of pesticides, accumulating a wide spectrum of pollutants, and significant correlations between pesticides and genetic damage in free-living rodents have already been detected.

The main aim of this study was the in situ assessment of Mancozeb genotoxicity, using two small mammals' species (*Mus spretus* and *Apodemus sylvaticus*) from a conventional agriculture (CA) versus a biological agriculture (BA) zone. It was found that exposure to a mixture of pesticide, where Mancozeb represents more than 50% of that mixture, has the ability to cause DNA damage in small mammals, assessed by comet, micronucleus and sperm abnormality assays. Moreover, both species, exposed to pesticides, with greater genetic damage, have also greater levels of Manganese. Thus, we can conclude that Mancozeb, the source of the highest levels of manganese found in small mammals, is the genotoxic agent at issue.

In conclusion, the present study clearly prove that conventional agriculture even with the use of pesticides legally approved in the European community, like Mancozeb, represent a higher genotoxic risk compared to biological agriculture. It is urgent to find and develop alternative and profitable agricultural practices, in order to minimize the use of pesticides, which represents a real global problem.

Key words: Mancozeb, Biomarkers of Genotoxicity, *Mus Spretus*, *Apodemus sylvaticus*, Mn quantification.

1. Introduction

The widespread use of pesticides is affecting the entire planet, including the human health [1-3] and conventional agriculture is largely responsible for the increased consumption of pesticides worldwide [4]. In Portugal, according to Eurostat [5], sales of pesticides have been increased in the last years and more than 17000 tones were sold in 2008.

Pesticides can be considered an economic, labor-saving, and efficient tool of pest management [6] and they are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment [7-9]. Even though the published results are not always in agreement with this and serious concerns have been raised about health risks resulting not only from occupational exposure to pesticides but also from pesticides residues on food, for the general population [10]. Consequently, in the last years, additional efforts have been produced to reduce the use of pesticides and biological agriculture can represent a valuable option, avoiding the use of pesticides. Nevertheless, it does not appear to have the potential to respond to the mass production of food to feed humanity [11] and it deserves wide experimentation to fully understand its potentialities, constraints and limitations [12].

Mancozeb, an ethylenebisdithiocarbamate (EBDC) is a widely used fungicide to protect fruits, vegetables and field crops against a large spectrum of fungal diseases. It is one of the most widely used commercial fungicides worldwide [13], mainly because of its low acute toxicity in mammals and scarce persistence in the environment [14]. Although, Mancozeb, mainly through its degradation product ethylenethiourea (ETU), has been reported to produce significant toxicological effects on thyroid [15, 16], immune system [17], reproductive system [18-21] and nervous system [22]. Moreover, Mancozeb was considered mutagenic [23], teratogenic [24] and a multipotent carcinogen, inducing a variety of tumors of different origin in rats after chronic exposure [25]. In addition, Mn, a coordinated transitional metal constituent of Mancozeb, is also a proven mutagen, causing DNA damage and chromosome aberrations [26].

The environmental genotoxicity of pesticides can be investigated in small mammals in the fields because they are able to accumulate a wide spectrum of pollutants which are present in the ecosystem [27] and, particularly, some significant

correlations between pesticides and genetic damage in free-living rodents have been detected [28]. Moreover, small mammals, mostly rodents, have enough genetic and physiological similarities with humans and, therefore, it allows extrapolating the effect of these pollutants.

The main aim of this study is the in situ assessment of pesticides genotoxicity, particularly Mancozeb, in two small mammal`s species from a conventional agriculture (CA) versus a biological agriculture (BA) zone. The wild small mammal species, Algerian mouse (*Mus spretus*, Lataste, 1883) and Wood mouse (*Apodemus sylvaticus*, Linnaeus, 1758) were chosen in this study as pesticide pollution bioindicators. These species have been widely used as a bioindicators in some environmental studies [29-33].

2. Material and Methods

2.1. Study Areas

This study was carried out in an agricultural zone of Alcobaça, district of Leiria, Portugal. In this region, agriculture is still an important economic activity with a wide area under agricultural practice. Animals were captured from conventional and biological agriculture zones. It was considered that these zones are both insert in the same biotope (≈1km of distance between them) and it was presumed that pesticide use is the main external factor that distinguishes them. The biological agriculture zone is correctly isolated from adjacent agriculture zones, so it is expected that it cannot be affected by adjacent pesticides applications.

Before animals capture, the agreement and the cooperation of the farmers involved were required and they were asked about pesticides and others chemical products that were used in the fields. While in biological agriculture (BA) zone, the owner farmer ensured that there were not used any type of chemical products, being an agricultural practice based on the use of natural enemies for the pest control, that was converted about 5 years ago, in conventional agriculture (CA) zone, the owner farmer guarantee that more than 50% of the applications were based on one EBDC: Mancozeb.

2.2. Mice Sampling and Sacrifice

A total of 46 small mammals of both sexes were captured between December 2010 and February 2011 in both zones. All the animals were treated according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [34]. Small mammals were captured with Sherman traps using a mixture of canned sardine, flour and oil as bait. Cotton was also placed inside each trap to minimize lower temperatures that were felt during the night. Pregnant or lactant females were discharged as well as animals with less than 10g of weight. Mice were housed in individual plastic cages until reach the laboratory with ad libitum access to food and water.

Animals were anesthetized and weighed before sacrifice. Cardiac puncture was done for blood collection, using a syringe with heparin to prevent coagulation

and, during dissection liver, brain,femurs and testis were removed. Livers and brains were stored at -70ºC before subsequent analysis.

2.3. Biomarkers of Genotoxicity

2.3.1. Comet Assay

The comet assay was performed as described by Singh et al. [35] with minor modifications. An aliquot of 15 µL of the blood samples collected by cardiac puncture was mixed with 300 µL 0.8% low-melting point agarose (37ºC) and then placed on a slide precoated with 1% normal-melting-point agarose. Immediately after that, the slide was covered with a coverslip and was then kept for 5 minutes on ice to solidify. After gently removing the coverslip, subsequent to solidification, slide was immersed in a cold lysing solution (2.5M NaCl, 0.1M EDTA, 10mM Tris, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) at 4ºC over night. After lysis, the slide was placed in a horizontal electrophoresis box. The box was filled with fresh electrophoresis solution (1 mM EDTA, 300 mM NaOH, pH≈13) and the slide was left in this solution for 40 min to allow unwinding. Electrophoresis was performed using the same solution at 25 V and 300 mA for 30 min. After that, the slide was gently neutralized with 0.4 M Tris buffer at pH 7.5 three times and then stained with SYBR Safe 4µL/mL. All steps described were performed under dark conditions to minimize additional ultraviolet induced DNA damage.

The slides were examined, and comet images were captured using a fluorescence microscope (Olympus BX41). Approximately 200 randomly selected cells were analyzed at 400x magnification per each animal. Extent of DNA migration was determined using a computerized image analysis system (Comet Score TM - TriTek Corp).

2.3.2. Micronucleus Assay using Acridine Orange

At the time of sacrifice, bone-marrow cells from both femurs of each animal were flushed with foetal calf serum, for the estimation of the frequency of micronucleated PCEs, according to Schmid [36]. The obtained cell suspension was centrifuged (800rpm, 5 min), the supernatant was removed and the pellet re-suspended in foetal calf serum. Then, a drop of the suspension was smeared on a

clean slide, air-dried, fixed in methanol for 10 minutes and stained with AO for fluorescent microscopic examination (Olympus BX41, equipped with blue excitation and 515-530 nm barrier filter) with a magnification of 400×. The frequency of MN in 1000 PCE per mouse (coded slides) was scored. The acridine orange (0.24mM in Sorensen Buffer pH=6.8) used was prepared from a stock solution of acridine orange 0.1% according to Krishna and Hayashi (2000) [37].

2.3.3. Sperm Abnormality Assay

The testis of the mice was dissected out, placed in 1 mL of Sorensen buffer (pH 7.0) and gently centrifuged (800rpm, 10 min) to obtain a pellet of undamaged cells. After removal of the supernatant, the pellet was re-suspended in 1 mL of Sorensen buffer. A drop of the suspension was placed on a clean slide and a smear is made, air-dried and fixed in absolute methanol for 10 min. After drying overnight the slides were stained with 10% Giemsa for 1 h [38] and observed under the microscope with a magnification of 100×. According to Wyrobek and Bruce [39], 1000 sperm per animal were assessed for morphological abnormalities, which included without hook, banana shape, amorphous, folded on themselves and two tails.

2.4. Determination of Manganese in the Liver

About 150 mg of each sample of liver pre-stored at -70ºC from 10 *Mus spretus* and 10 *Apodemus sylvaticus* randomly selected from both zones (5 from CA zone and 5 from BA zone) were defrosted and dried at 80ºC until reaching a constant weight. After that, dried samples were digested with 5 mL of 65% suprapure nitric acid during 3 hours at 150ºC.

The resulting clear acid solutions were transferred to 50mL volumetric flasks that were completed with deionized water. These solutions were used for the determination of Mn concentrations that were determined by Graphite Furnance Atomic absorption spectroscopy (GFAAS) with a PerkinElmer AAnalyst™ 700 atomic absorption spectrometer equipped with an HGA Graphite Furnace and a programmable sample dispenser (AS 800 Auto Sampler and WinLab 32 for AA software). Mg(NO3)2·6 H2O (0.84 mol/L) was used as a chemical modifier. Samples were measured at least twice and every measurement consisted of two separate injections into the graphite furnace.

Results were expressed as micrograms of Mn per gram of dry liver. Calibration curves were automatically obtained by the device starting from a 25µg/L solution of MnCl2 that was successively diluted for 12.5, 6.3, and 2.5 µg Mn/L. The limit of detection was 0.05 µg Mn/L.

2.5. Statistical Analysis

The statistical analysis of the two species captured was done separately because of the differences between them. The data were statistically analyzed using SPSS v.19.0. All variables were checked for normal distributions, using Kolmogorov-Smirnov test (Lilliefors significance correction).

Differences between conventional agriculture group and biological agriculture group were assessed with Student's t-test if they exhibited a normal distribution; if not, the Mann-Whitney`s U-Test was used instead. Differences between males and females of each zone were always assessed. The significance of differences was examined at the p-value of 0.05.

3. Results

3.1. Comet Assay

There were no significant differences between males and females of each zone, thus sexes were combined for statistical analysis. % tail DNA was not normally distributed for *Mus spretus* (D=0.233, p=0.038) and *Apodemus sylvaticus* (D=0.286, p=0.033) samples from CA zone.

As we can see in Figure 1, for both species there is a tendency for greater genetic damage in animals from CA zones, which are exposed to pesticides. However, there is a significant difference between CA zone (Mean % tail DNA=9.80±2.48) and BA zone (Mean % tail DNA=6.96±2.63) only for *Apodemus sylvaticus* (U=16.00, p=0.031). *Mus spretus* from CA zone (Mean % tail DNA=8.10±3.04) and *Mus spretus* from BA zone (Mean % tail DNA=6.96±2.63) do not show significant differences (U=85.00, p=0.550) relatively to the DNA damage estimated by % tail DNA.

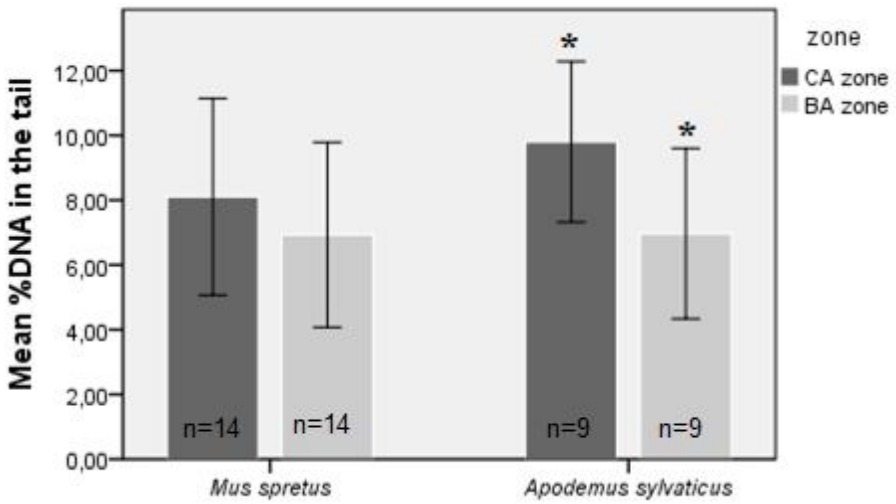

Figure 1. DNA damage assessed by mean of % DNA in the tail of the lymphocyte comets from small mammals of the different groups analyzed. Error bars represent the standard deviation of the mean (*p<0.05 by Mann-Whitney`s U-Test, significant differences between CA and BA zones; CA zone-Conventional agriculture zone; BA zone-Biological agriculture zone; n= number of tested animals).

3.2. Micronucleus Assay

There were no significant differences for MNPCEs number between males and females of each zone, thus sexes were combined for statistical analysis. MNPCEs number was not normally distributed for *Mus spretus* from CA (D=0.252, p=0.0316) and BA (D=0.227, p=0.048) zones.

Table 1. Frequency of micronucleated polychromatic erythrocytes (MNPCE) in 1000 polychromatic erythrocytes from bone-marrow cells.

	Mean ± SD			
	Mus spretus		***Apodemus sylvaticus***	
	CA zone	BA zone	Ca zone	BA zone
number of MNPCEs	6,21±2,26*	2,07±2,17*	3,22±1,56	2,11±1,62

*p<0.05 by Mann-Whitney`s U-Test; significant differences between BA and CA zones (BA-Biological agriculture; CA-Conventional agriculture; SD-Standard deviation; MNPCE-Micronucleated polychromatic erythrocytes; n= Number of tested animals).

As we can see in Table 1, for both species there is a tendency for greater genetic damage in animals from CA zones, which are exposed to pesticides. However, there is a significant difference between CA (Mean % tail DNA=9.80±2.48) and BA zones (Mean % tail DNA=6.96±2.63) only for *Mus spretus* (U=14.00, p<0.001). *Apodemus sylvaticus* do not show significant differences (t=1.482, p=0.158) relatively to the DNA damage estimated by MN PCEs number, between the two zones.

3.3. Sperm Abnormality Assay

In this assay different morphological alterations in the mice sperm were observed. Amorphous sperm and sperm without the usual hook are the most frequently observed alterations. On the contrary, the two tails and the banana form are the least common morphological abnormalities (Table 2).

Abnormal sperm frequency was normally distributed for all the samples and it increased significantly in *Mus spretus* from CA zone when compared with Mus spretus from BA zone (t=3.201, p=0.006). So, small mammals exposed to pesticides

have increased abnormal sperm. *Apodemus sylvaticus* from CA zone also present a higher frequency of abnormal sperm compared to BA zone but this result is not statistically significant (t=0.500, p=0.638).

Table 2. Frequency of different types of sperm abnormality and the total abnormal sperm.

	Mean ± SD			
	Mus spretus		***Apodemus sylvaticus***	
	CA zone n=8	BA zone n=9	Ca zone n=6	BA zone n=2
Hook loss (%)	3.20±1.29	1.46±0.81	1.12±0.67	0.50±0.00
Banana (%)	0.96±0.59	0.58±0.40	0	0
Amorphous (%)	2.71±2.24	1.00±0.50	0.94±0.36	0.90±0.42
Two tails (%)	0.025±0.046	0.03±0.1	0.10±0.10	0.35±0.21
Total abnormal sperm (%)	6.9±3.29*	3.07±1.39*	2.16±1.09	1.75±0.21

*****p<0.05 by Student`s T-Test; significant differences between BA and CA zones (BA- biological agriculture; CA- conventional agriculture; SD-standard deviation).

3.4. Determination of Manganese in the Liver

Mn concentrations in the liver were normally distributed for all the samples. Both *Mus spretus* and *Apodemus sylvaticus* from CA zone had statistically significant high levels of Mn in the liver compared to animals from BA zone (Figure 2).

For *Mus spretus*, animals from CA zone had 7.20±1.87 µg Mn/g dry liver, while animals from BA zone had 4.21±1.09 µg Mn/g dry liver (t=3.078 p=0.015). For *Apodemus sylvaticus*, animals from CA zone had 7.65±1.12 µg Mn/g dry liver, while animals from BA zone had 4.85±1.83 µg Mn/g dry liver (t=2.915 p=0.019).

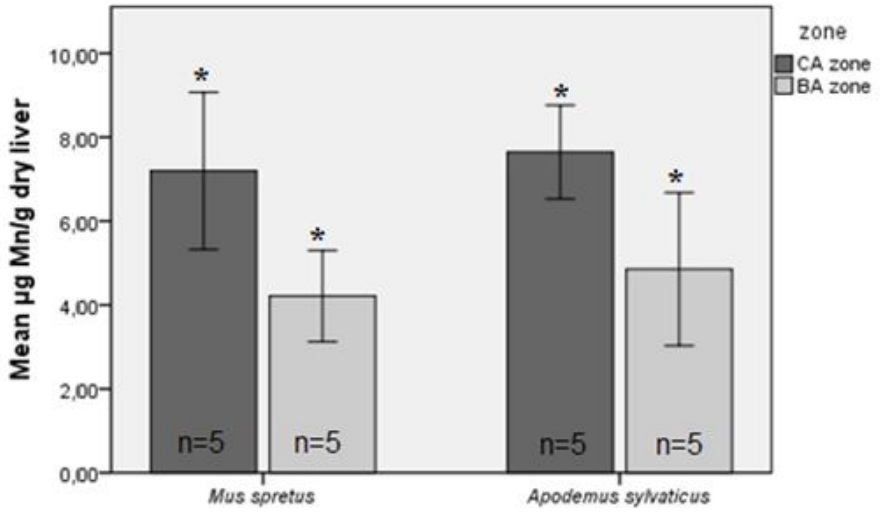

Figure 2. Mn quantification from small mammal´s livers of the different groups analyzed. Error bars represent the standard deviation of the mean (*p<0.05 by Student´s T-Test, significant differences between CA and BA zones; CA zone-Conventional agriculture zone; BA zone-Biological agriculture zone; n= number of tested animals).

4. Discussion of Results

Pesticides are widely used in modern agriculture despite their hazards to humans and to nature. They have played a key role in providing reliable agricultural products at prices affordable to consumers, improving the quality of produce, and ensuring high profits to farmers. Theoretically, they are developed to function with minimal impacts on human health and the environment. At European level, according to 91/414/EEC Directive (Council Directive 91/414/EEC, 1991), active substances in pesticides formulations are only approved for use if they have undergone a peer-reviewed safety assessment [8]. Despite of that, serious concerns have been raised about health risks resulting from exposure to these approved pesticides and numerous studies have been published in order to alert about this [40-42].

In particular, the low reported acute toxicity and short environmental persistence of Mancozeb have allowed its worldwide usage in agriculture, even though numerous effects of its chronic exposure have been reported. In fact, as stated before, Mancozeb has the capability to alter the function of thyroid [15, 16], immune system [17], reproductive system [18-21] and nervous system [22]. Moreover, in a long-term experimental study, Belpoggi *et al.* (2002) verified that Mancozeb is a multipotent carcinogen inducing a variety of tumors of different origins in rats [25]. Nevertheless, Mancozeb is included in the list (referred to as Annex I of the Council Directive 91/414/EEC, 1991) of active substances that are considered acceptable in regard to their impact on the environment, human and animal health, and therefore authorized for use in pesticide formulations within the European Community [8].

The present study clearly demonstrates that exposure to a mixture of pesticide, where Mancozeb represents more than 50% of that mixture, has the ability to cause DNA damage in small mammals, assessed by comet, MN and sperm abnormality assays. These results are in accordance with other studies using comet assay [23], MN assay [43] as well as the sperm abnormality assay [44] to assess Mancozeb genotoxicity.

To guarantee that Mancozeb, the most widely used pesticide in the CA zone, was the genotoxic agent responsible to the DNA damage detected, it would be necessary to prove that these animals were really exposed. Mancozeb is an EBDC with the presence of coordinated transitional metals and ETU is the specific

metabolite of these compounds resulting from a spontaneous breakdown. Therefore, ETU quantification in urine has been widely used as a biomarker of exposure to Mancozeb and others EBDCs [45]. However, in this study, it was not possible to use this biomarker to assess exposure to Mancozeb because the amounts of urine obtained from small mammals were not sufficient to quantify ETU. To achieve this, it would be necessary to keep animals in metabolic cages for several days, which was not possible.

Alternatively, it was used Mn quantification by GFAAS to indirectly quantify Mancozeb inside the animal's body. Mancozeb is chemically characterized by the presence of coordinated transitional metals in the molecule, like Mn and Zn. Thus, measurement of these metals in biological tissues has been proposed as an alternative approach to monitor Mancozeb exposure. This application may be very useful for biological monitoring, since determination of metals can be performed with very sensitive AAS methods which are of lower complexity than those required for metabolite determination, like ETU [14]. Canossa et al. (1993) have already observed an increased urinary excretion of manganese in workers exposed to Mancozeb [46] but more research is needed because the available data, at present, are not sufficient to validate the use of metals as biomarkers of exposure to Mancozeb [14]. In addition, it is necessary to investigate the presence of metals not only in urine but also in other tissues and biological fluids as potential biomarkers.

Consequently, in the present study, it was used Mn quantification by GFAAS in small mammals' liver as a potential biomarker of Mancozeb exposure, because the liver is a filter organ where metals will be accumulated. As a result, it was found that both species (*Mus spretus* and *Apodemus sylvaticus*) from CA zone, exposed to pesticides, with greater genetic damage, have also greater levels of the metal Mn, comparing to animals from BA zone. So, considering that Mancozeb is the widely used pesticide in the CA zone, we can conclude that this pesticide will be the source of the highest levels of Mn found in small mammals. In other words, it seems that Mancozeb is the genotoxic agent which caused the DNA damage detected.

Despite the evident genotoxic action of Mancozeb, the two analyzed species seems to be affected in a different away. While, for *Mus spretus* significant genetic differences were detected by the MN and sperm abnormality assays, for *Apodemus sylvaticus* significant genetic differences between animals from CA and BA were only detected by comet assay. Comet, MN and sperm abnormality assays assess the

DNA damage in different cells populations: comet assay evaluate the DNA damage in lymphocytes from the bloodstream, MN were detected in PCEs from bone marrow and sperm abnormalities were assessed in male germ cells. Thus, our results suggest that the two species subject to the same genotoxic agents seem to have populations of cells to be affected differently.

The differential response to the same genotoxic agent may be due because these two species have very different habits of life, including different feeding habits. *Mus spretus* are exclusively herbivores, whereas *Apodemus sylvaticus* are more omnivorous and their diets may include a large fraction of arthropods. Thus, the intensity and the frequency of the animal`s exposure to pesticides will be different in the two species because different foods will have different levels of these chemicals. On the other hand, different responses to genotoxic agents may only reflect intrinsic differences of species rather than differences related to exposure that was already corroborated in previous studies [47, 48].

It would be expected that Comet assay was a more sensitive test than MN assay because there are a lot of studies that confirm the ability of comet assay to detect the effect of genotoxic agents at lower doses than the MN assay. However, taking into account the present study, it seems that this sensibility depend on the species concerned. For Mus spretus, MN assays was the ability to detect significant differences in DNA damage between small mammals from CA and BA zones that were not detected by Comet Assay. MN assay using AO as staining method is more useful and gives more reliable results than the usual Giemsa staining because both PCEs and a MN can be easily distinguished. So, MN assay using AO staining has also a good sensibility that should be considered.

On the other hand, the sensitivity difference between the comet assay and MN assay may be due to their different endpoints. MN assay determines unrepaired DNA strand breaks, while the comet assay determines strand breaks (single and/ or double) and labile sites that are subsequently removed by repair enzymes [49]. Comet assay can determine the short-lived DNA damage while the MN assay detects the structural and numerical chromosomal damage [37, 50]. Thus, like several reports suggests, a combination of this two assays might therefore be recommended to understand the mechanisms underlying mutagenicity and to improve the sensibility as well as reliability of detection which can better facilitate the risk assessment process [51].

Furthermore, a combination of these two assays with the sperm abnormality assay is also very useful because it is a fast and sensitive mutagenicity test [39] used to detect cytogenetic damage in germ cells further than somatic cells. However, the classification used in this test is somewhat subjective with many technical artifacts and, therefore, it has not been widely used in recent times. Nevertheless, we find significant differences in the percentage of sperm abnormalities in Mus spretus from CA zone, comparing to BA zone, but there were no significant differences for Apodemus sylvaticus. It is important to refer that this result can be due to the reduced number of males Apodemus sylvaticus captured: only six males in CA zone and two males in BA zone. In fact, a larger sampler would be essentially for more reliable results not only for sperm abnormality assay but also for the other genotoxic tests, especially in wild species like these because the variability among animals can be higher than in laboratory animals.

Our study clearly showed that the use of only one cytogenetic end point is not sufficient to understand the range of the effects of pesticides on the genetic material in an in situ study. However, when other end points are included like in the present study, the method can be very promising. Moreover, because of differences in sensitivities, it is important to have more than one species for comparison to establish a genotoxic profile of one area.

In situ studies like the present that compares bioindicator species from a conventional agriculture zone versus a biological agriculture zone can be extremely important in assessing the toxicity of pesticides to humans. Even though the general population is not as exposed as animals because they only contact to pesticides through the consumption of agricultural products while animals live in the fields and are directly subjected to pesticide applications, the evaluation of the toxicological risk of pesticides in this way for humans is quite acceptable and more realistic than animal laboratory experiments. In fact, it is known that many pesticides are transformed in the environment through physical, chemical and biological processes which are intended to detoxify them but often the transformation process forms products that are more toxic than the parent [52]. Moreover, it is not feasible to predict the toxicity of pesticide mixtures on the basis of the results of the toxicity of single components because the effects of a mixture of pesticides are often different from the individual effects of a single components [53] and human exposure is rarely

limited to a single compound. So, in situ studies are obviously closer to the real situation and must be considered in the pesticide approval process.

In conclusion, the present study clearly prove that conventional agriculture even with the use of pesticides legally approved in the European community, like Mancozeb, represent a higher genotoxic risk compared to biological agriculture. Despite all the controversy, biological agriculture has been adopted by an increasing number of farmers, like the farmer who collaborated in this study, and they believe that this practice has the potential for mass production of the amount of calories needed to feed humanity. We do not know if this is possible but it is urgent to find and develop alternative and profitable agricultural practices, in order to minimize the use of pesticides, which represents a real global problem.

5. Final Remarks

• The present work contributes to alert about hazard effects resulting from pesticide exposure, particularly Mancozeb, a pesticide legally authorized for use in European community;

• As revealed by the three cytogenetic endpoints used, Mancozeb is capable of inducing DNA damage in *Mus spretus* and *Apodemus sylvaticus* species;

• The use of several tests is essential to have a full picture of the genotoxic effects especially in wild species since the variability among animals can be higher than in laboratory animals;

• The use at least two sympatric species to detect the presence of genotoxic agents is essential because there may be a difference in sensitivity of the species selected as bioindicators in relation to the cytogenetic end points analyzed;

• Mn quantification seems to be a good biomarker of exposure to Mancozeb and more research is needed to validate the metal quantifications as biomarkers of exposure to this type of pesticides;

• The present results provide reference values for the analyzed biomarkers allowing future comparisons with other small mammals' populations.

• *Mus spretus* and *Apodemus sylvaticus* are suitable bioindicator species of pesticide pollution, demonstrating variation in morphological, biochemical and genotoxic effects of exposure;

• Finally, despite Mancozeb toxicity, it will belong to the most widely used pesticide in the world due to their efficacy, and low relatively low cost and persistence in the environment. As a result, a large part of the population will continue to be exposed, and concerns for their known and potentially newly discovered adverse effects will remain alive.

6. References

[1] G.Y.H. McClure, R.M. Helm, K. Stine, A.W. Burks, S.M. Jones, J. Gandy, Evaluation of immune parameters in propanil-exposed farm families, Archives of Environmental Contamination and Toxicology, 41 (2001) 104-111.

[2] R. McKinlay, J.A. Plant, J.N.B. Bell, N. Voulvoulis, Calculating human exposure to endocrine disrupting pesticides via agricultural and non-agricultural exposure routes, Science of The Total Environment, 398 (2008) 1-12.

[3] A. Boobis, B. Ossendorp, U. Banasiak, P. Hamey, I. Sebestyen, A. Moretto, Cumulative risk assessment of pesticide residues in food, Toxicology Letters, 180 (2008) 137-150.

[4] A.L. Aspelin, Pesticides industry sales and usage: 1994-95 market estimates, United States Environmental Protection Agency, Washington, 1997. Available from: http://www.epa.gov/opp00001/pestsales/95pestsales/market_estimates1995.pdf. Accessed July 2011.

[5] Eurostat, Statistical Office of the European Communities, Luxemburg, 2011. Hyperlink to the graph: http://epp.eurostat.ec.europa.eu/tgm/graph.do?pcode=tag00084&language=en. Accessed July 2011.

[6] J. Cooper, H. Dobson, The benefits of pesticides to mankind and the environment, Crop Protection, 26 (2007) 1337-1348.

[7] WHO, International Code of Conduct on the Distribution and Use of Pesticides: Guidelines for the Registration of Pesticides, Rome, 2010.

[8] Commission of the European Communities, Council Directive 91/414/EEC of 15 July 1991, Concerning the Placing of Plant Protection Products on the Market, Official Journal L 230, Brussels, 1991.

[9] EPA, Registering Pesticides, 2009. Available from: http://www.epa.gov/pesticides/regulating/index.htm. Accessed july 2011.

[10] C. Bolognesi, G. Morasso, Genotoxicity of pesticides: potential risk for consumers, Trends in Food Science & Technology, 11 (2000) 182-187.

[11] F. Carvalho, Agriculture, pesticides, food security and food safety, Environmental Science & Policy, 9 (2006) 685-692.

[12] T. Gomiero, D. Pimentel, M.G. Paoletti, Is there a need for a more sustainable agriculture?, Critical Reviews in Plant Sciences, 30 (2011) 6-23.

[13] EPA, Reregistration Eligibility Decision for Mancozeb, 2005.

[14] M. Maroni, C. Colosio, A. Ferioli, A. Fait, Biological Monitoring of Pesticide Exposure: a review, Toxicology, 143 (2000) 1-118.

[15] R. Kackar, M.K. Srivastava, R.B. Raizada, Induction of gonadal toxicity to male rats after chronic exposure to mancozeb, Industrial Health, 35 (1997) 104-111.

[16] K. Steenland, L. Cedillo, J. Tucker, C. Hines, K. Sorensen, J. Deddens, V. Cruz, Thyroid hormones and cytogenetic outcomes in backpack sprayers using ethylenebis(dithiocarbamate) (EBDC) fungicides in Mexico, Environmental Health Perspectives, 105 (1997) 1126-1130.

[17] E. Corsini, S. Birindelli, S. Fustinoni, G. De Paschale, T. Mammone, S. Visentin, C.L. Galli, M. Marinovich, C. Colosio, Immunomodulatory effects of the fungicide Mancozeb in agricultural workers, Toxicology and Applied Pharmacology, 208 (2005) 178-185.

[18] P.N. Baligar, B.B. Kaliwal, Induction of gonadal toxicity to female rats after chronic exposure to mancozeb, Industrial Health, 39 (2001) 235-243.

[19] G. Rossi, R. Buccione, M. Baldassarre, G. Macchiarelli, M. Palmerini, S. Cecconi, Mancozeb exposure in vivo impairs mouse oocyte fertilizability, Reproductive Toxicology, 21 (2006) 216-219.

[20] G. Rossi, M. Palmerini, G. Macchiarelli, R. Buccione, S. Cecconi, Mancozeb adversely affects meiotic spindle organization and fertilization in mouse oocytes, Reproductive Toxicology, 22 (2006) 51-55.

[21] B.B. Bindali, B.B. Kaliwal, Anti-implantation Effect of a Carbamate Fungicide Mancozeb in Albino Mice Industrial Health, 40 (2002) 191-197.

[22] L. Miranda-Contreras, R. Dávila-Ovalles, P. Benítez-Díaz, Z. Peña-Contreras, E. Palacios-Prü, Effects of prenatal paraquat and mancozeb exposure on amino acid synaptic transmission in developing mouse cerebellar cortex, Developmental Brain Research, 160 (2005) 19-27.

[23] G. Calviello, E. Piccioni, A. Boninsegna, B. Tedesco, N. Maggiano, S. Serini, F.I. Wolf, P. Palozza, DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: Involvement of the oxidative mechanism, Toxicology and Applied Pharmacology, 211 (2006) 87-96.

[24] B.P. Varnagy, E. Molnar, I. Takacs, A. Karpati, Interaction of dithane M-45(mancozeb) and lead acetate during a teratogenicity test in rats, Acta Veterinaria Hungara, 48 (2000) 113-124.

[25] F. Belpoggi, M. Soffritti, M. Guarino, L. Lambertini, D. Cevolani, C. Maltoni, Results of Long-Term Experimental Studies on the Carcinogenicity of Ethylene-bis-Dithiocarbamate (Mancozeb) in Rats, Annals of the New York Academy of Sciences, 982 (2002) 123-136.

[26] G.B. Gerber, A. Léonard, P. Hantson, Carcinogenicity, mutagenicity and teratogenicity of manganese compounds, Critical Reviews in Oncology/Hematology, 42 (2002) 25-34.

[27] S.S. Talmage, B.T. Walton, Small mammals as monitors of environmental contaminants, Review of Environmental Contamination and Toxicology, 119 (1991) 47-108.

[28] K. McBee, J.W. Bickham, Petrol-chemical related DNA damage in wild rodents detected by flow cytometry, Bulletin of Environmental Contamination and Toxicology 40 (1988) 343-349.

[29] L.A. Ieradi, S. Moreno, J.P. Bolívar, A. Cappai, A. Di Benedetto, M. Cristaldi, Free-living rodents as bioindicators of genetic risk in natural protected areas, Environmental Pollution, 102 (1998) 265-268.

[30] L.A. Ieradi, M. Cristaldi, D. Mascanzoni, E. Cardarelli, R. Grossi, L. Campanella, Genetic damage in urban mice exposed to traffic pollution, Environmental Pollution, 92 (1996) 323-328.

[31] C. Tanzarella, F. Degrassi, M. Cristaldi, S. Moreno, A. Lascialfari, G. Chiuchiarelli, L.A. Ieradi, Genotoxic damage in free-living Algerian mouse (Mus spretus) after the Coto Doñana ecological disaster, Environmental Pollution, 115 (2001) 43-48.

[32] A.C. Nunes, M. da Luz Mathias, A.M. Crespo, Morphological and haematological parameters in the Algerian mouse (Mus spretus) inhabiting an area contaminated with heavy metals, Environmental Pollution, 113 (2001) 87-93.

[33] A. Gorriz, S. Llacuna, M. Riera, J. Nadal, Effects of air pollution on hematological and plasma parameters in Apodemus sylvaticus and Mus musculus, Archives of Environmental Contamination and Toxicology, 31 (1996) 153-158.

[34] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for experimental purposes, Official Journal of the European Union. Available from: http://ec.europa.eu/food/animal/welfare/references_en.htm. Accessed July 2010.

[35] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Experimental Cell Research, 175 (1988) 184-191.

[36] W. Schmid, The micronucleus test, Mutation Research, 31 (1975) 9-15.

[37] G. Krishna, M. Hayashi, In vivo rodent micronucleus assay: protocol, conduct and data interpretation, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 455 (2000) 155-166.

[38] U.R. Acharya, R.M. Rathore, M. Mishra, Role of vitamin C on lead acetate induced spermatogenesis in swiss mice, Environmental Toxicology and Pharmacology, 13 (2003) 9-14.

[39] A.J. Wyrobek, W.R. Bruce, Chemical induction of sperm abnormalities in mice, Proceedings of the National Academy of Sciences, 72 (1975) 4425-4429.

[40] C.A. Damalas, I.G. Eleftherohorinos, Pesticide Exposure, Safety Issues, and Risk Assessment Indicators, International Journal of Environmental Research and Public Health, 8 (2011) 1402-1419.

[41] C.A. Damalas, Understanding benefits and risks of pesticide use, Scientific Research and Essay, 4 (2009) 945-949.

[42] A.J. Karabelas, K.V. Plakas, E.S. Solomou, V. Drossou, D.A. Sarigiannis, Impact of European legislation on marketed pesticides - A view from the standpoint of health impact assessment studies, Environment International, 35 (2009) 1096-1107.

[43] A. Medjdoub, S.A. Merzouk, H. Merzouk, F.Z. Chiali, M. Narce, Effects of Mancozeb and Metribuzin on in vitro proliferative responses and oxidative stress of human and rat spleen lymphocytes stimulated by mitogens, Pesticide Biochemistry and Physiology, 101 (2011) 27-33.

[44] P.K. Khan, S.P. Sinha, Ameliorating effect of vitamin C on murine sperm toxicity induced by three pesticides (endosulfan, phosphamidon and mancozeb), Mutagenesis, 11 (1996) 33-36.

[45] C. Aprea, C. Colosio, T. Mammone, C. Minoia, M. Maroni, Biological monitoring of pesticide exposure: a review of analytical methods, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 769 (2002) 191-219.

[46] E. Canossa, G. Angiuli, G. Garasto, A. Buzzoni, E. De Rosa, Dosage indicators in farm workers exposed to mancozeb, La Medicina del lavoro, 84 (1993) 42-50.

[47] A.M.S. Bueno, C.A.d.B. Pereira, M.N. Rabello-Gay, Environmental genotoxicity evaluation using cytogenetic end points in wild rodents, Environ Health Perspect, 108 (2000).

[48] U.A. Meyer, U.M. Zanger, Molecular mechanisms of genetic polymorphisms of drug metabolism, Annual Review of Pharmacology and Toxicology, 37 (1997) 269-296.

[49] V.D. Heuser, V.M. de Andrade, A. Peres, L.M. Gomes de Macedo Braga, J.A. Bogo Chies, Influence of age and sex on the spontaneous DNA damage detected by Micronucleus test and Comet assay in mice peripheral blood cells, Cell Biology International, 32 (2008) 1223-1229.

[50] F. Van Goethem, D. Lison, M. Kirsch-Volders, Comparative evaluation of the in vitro micronucleus test and the alkaline single cell gel electrophoresis assay for the detection of DNA damaging agents: genotoxic effects of cobalt powder, tungsten carbide and cobalt-tungsten carbide, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 392 (1997) 31-43.

[51] I. Kim, C. Hyun, Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms, Ecotoxicology and Environmental Safety, 64 (2006) 288-297.

[52] C.J. Sinclair, A.B.A. Boxall, Assessing the Ecotoxicity of Pesticide Transformation Products, Environmental Science & Technology, 37 (2003) 4617-4625.

[53] M. Marinovich, F. Ghilardi, C.L. Galli, Effect of pesticide mixtures on in vitro nervous cells: Comparison with single pesticides, Toxicology, 108 (1996) 201-206.